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Ameliorative Potential of Ethanolic Garlic Extract Against Acetaminophen Induced Morphometric and Morphological Changes in Mice (Albino) Embryo

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Abstract
Acetaminophen is a widely used drug for pain and common fevers during pregnancy. The study was aimed to investigate the acetaminophen (N-acetyl-para-aminophenol) induced Embryotoxicity in female Albino Mice and ameliorative potential of garlic (Allium sativum) extract. For this purpose, phytochemical 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 Morphological abnormalities like hemorrhagic spots, deformed axis, micromelia, macrotia, club feet, skin lesions and open eyelid. Co-treatment of Ethanolic Garlic Extracts (EGE) as ameliorant reduced these malformations across all the treatment groups (D-G). Whereas, Morphometric parameters showed that fetus's weight, hind limb, fore limb, tail and snout length, crown rump, and head circumference decreased significantly (p<0.05) in Acetaminophen treated groups (B and C) as compared to control (A). Whereas, ethanolic garlic extract as curative agent reduced the toxicant effects of acetaminophen in groups (D-G). This amelioration showed occurrence of some biological active compounds in the EGE like an amino acid derivative NAC (N-Acetyl Cysteine) which might prove to be protective agent in acetaminophen caused teratogenicity.

Keywords: *Acetaminophen, Ethanolic garlic extract, embryotoxicity, N-Acetyl Cysteine, Developmental abnormalities.*

Introduction

It is common practice of many underprivilege countries to use drugs for common flu and fever without prior prescription, frequent use of over the counter (OTC) drugs have reported negative effects according to recent medical research. Acetaminophen (commonly sold as Paracetamol, Tylenol and Calpol) is used for many years for pain management and fevers. It is reported to show Acute liver failure and Neurological altercations in patients. Many medical professionals and scientists are now collaborating to find the underlaying mechanism of action. (Chun et al., 2009; Bunchorntavakul and Reddy 2013; Michautet al., 2014).

Health care professionals are keener on more up to date information about this medication, specially concerning with complex physiological phenomenon like pregnancy. There is lack of information in toxicity profiles for such cases (Feinstein et al., 2000; Zhang et al, 2009).

Earlier form of Acetaminophen is Aspirin, that has a history of more than 100 years and is commonly associated with Rye's Syndrome of children. It is widely consumed during pregnancy that leads toward a ban of its usage. Conversely, the use of Acetaminophen has significantly increased in pregnant women due to its analgesic nature, which is corelated with stark rise in attention-deficit/hyperactivity disorder (ADHD), and developmental abnormalities in offspring (Eder et al., 2006).

Acetaminophen has established itself as a safer option to be used in common pain and fever management during pregnancy around many parts of the world, however overdosing due to no prescription is noted by many medical practitioners. This is mainly due it's over the counter (OTC) availability. Also, many patients with arthritis are often treated with high dose of acetaminophen (Zhang et al., 2009). Independent research of medical science in 2006 reported a specialized experiment during which several young volunteers ingested normal doses of acetaminophen to ascertain its effects in normal physiology. They excrete large amount of transaminase that indicated toxicity of liver pathway in metabolism. More research efforts are diverted to study the metabolic pathway pf this drug (Watkins et al., 2006). Several cases of childhood asthma were reveled in newborns, in which the mothers took acetaminophen for general pain management, a specialized 6-month study was made that made correlation with this effect. Health of fetus in this regard is huge cause of concern (Perskyet al., 2008).

Acetaminophen metabolism produces a harmful intermediate i.e. (N-acetyl-p benzoquinone imine) it is produced by kidney and livers cells via cyp450 co-enzymes pathway, and cause toxicity in liver cells if not detoxified in time, Glutathione is able to remove these harmful metabolites (Moynihan, 2002; Lee, 2004).

Garlic (Allium sativum) belongs to group of plants that are bulbous in nature (Gunther., 2013) and is related to onion plant family members, it is present in all over the world, but mainly cultivated in Central and South east Asia due to its large-scale effects, including not only its flavored properties as spice in foods, but also the medicinal advantages in various pharmaceutical industries (Van Wyk and Wink, 2018). Garlic raw form contains many Sulphur containing chemicals that have important implications in human dietary needs, such as VINLYLDITHIINS, ALLCIN, S-ALLYLCYSTEINE, DIALLYLPOLYSULFIDES, and N-ACETYLCYSTEINE. These are group of organo-sulphur compounds that constitutes important enzymes and flavonoids, the pungent smell of crushed garlic compound is due to Sulphur containing products that are released and their breakdown produced characteristic garlic smell (Jones et al., 2004).

Glutathione reserves in livers cells are important to maintain the detoxification abilities, precursor of glutathione is N-Acetylcysteine (NAC), not only helps in controlling liver injury but also supports the proper blood flow and oxygen consumption (Harrisonet al.,1991). It is proven that garlic extract is loaded with amino acid derivates like N-Acetylcysteine (Asdaqet al., 2011) and recent medicinal reports exhibit its ability to restore glutathione reserves in acetaminophen prenatal exposure.

Our Present Studies was conducted to Evaluate the Acetaminophen induced embryotoxicity in albino mice (Mus musculus) and curative efficacy of Ethanolic Garlic Extract (Allium sativum).

Materials And Methods

Handling of Animals

Procedures and study protocols are duly approved by National Bioethics Committee (NBC) of Pakistan with the prior permission granted by Ethical committee of Institute of Zoology, Bahhaudin Zakarya University, Multan, Pakistan with Ref.No. Zool. 109, Date: 10-11-2023. Standard room temperature was maintained at 25c0 at Animal house BZU Multan, Pakistan for rearing and breeding of mice. Feed No.14 (National feeds Ltd) it was administered to animals in form of pallets. A wood chip bedding is used for placing the mice in speciated cages. Tagging was used for observations and record keeping of animals.

Animal mating and gestation

7-8weeks old albino mice (Mus musculus) were used in this experiment. Initial body weight was in range of 20-25g for all mice used. They were reared in Animal house BZU, Multan, Pakistan. For pregnancy a timed mating process was used as per previous reports of (Rodriguez et al.,2016). Animals were placed with (1:2) of male to female respectively. Indication of successful mating is a vaginal plug, that can be seen after copulation, and this was named as gestation day zero "0"

Identification and collection of plant sample

Garlic bulbs were taken from Botany division, biopark, BZU, Multan, Pakistan. Garlic plant was verified by plant taxonomist. Distilled water was used for peeling. Small blocks of equal sizes were obtained and air dried for 8 days at standard room temperature i.e. 25c0 to prevent denaturation of sample. Soxhlet's apparatus was used for extract preparation. 300-400g of garlic powder was extracted with ethanol solvent mixture. A rotary evaporator was used at 40c0 to obtain plant extract. Small vials were used to store the extract. Distilled water was used to adjust concentrations when required (Velega et al., 2017)

Phytochemical Analysis of Garlic Extracts

Phytochemical analysis of Garlic (*Allium sativum*) for the presence or absence of phytochemicals was carried using standard procedures given in reports of Santhi and Sengottuvel (2016) and Adawia et al. (2016)

Toxicant Administration with test Ameliorative agent

Acetaminophen as toxicant, co-treated with Garlic extract, were given to pregnant albino mice (Mus Musculus). Different doses of Acetaminophen 150mg/kg/B.W and 300mg/kg/B.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). Acetaminophen (APAP) and Ethanolic Garlic extract (EGE) was co-administered orally with following doses as given below, EGE was given 1hour before Acetaminophen (APAP) administration in all experimental groups.

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. Garlic dose concentrations for extracts were chosen according to previously published report of (Saleh et ali., 2018).

Dissection and fetal recovery

All Pregnant female mice were anaesthetized by using chloroform. Standard surgical incision protocols were used for embryo sampling from females. Embryo weight was measure using digital machine (Schimadzu Ltd., Japan). 5% formalin solution was used to fix fetus and used for morphometric and morphological studies (Carson & Hladik, 1997)

Morphometric Studies

Morphometric parameters were noted using a laboratory grade digital vernier caliper up to 0.01mm precision, seven perimeters were recorded for analysis i.e. fetal body weight (mg), crown–rump length (mm), tail length (mm), snout length (mm), forelimb length (mm), hindlimb length (mm), and head circumference (mm) as per previous published reports of (Ali et al., 2021)

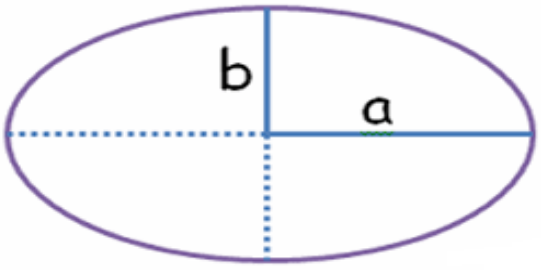


Figure No.1: For the measurement of head circumference (a= width, b= front)

Online Ellipse circumference calculator was used to calculate the head circumference of each fetus separately given below.
Circumference formula used in study= $2\pi 1/2(a2+b2)$
where $\pi = 3.14$, a= width of head (half diameter) and b= half of length (head)

Morphological Studies

All recovered fetuses are counted for gross viability, Morphological assessment was conducted with the help of a stereomicroscope (leica EZ4, Germany) that is used to detect physical malformations caused by the drug treatment, this includes limb deformities, craniofacial issues, axial skeletal abnormalities, spots of hemorrhage or skin lesions, for further comparative analysis and record, image J software was used to improve optimization and details in pictures.

Statistical Analysis

One way analysis of variance (ANOVA) was used for analytical studies via SPSS software (version 32), 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

PHYTOCHEMICAL ANALYSIS OF ETHANOLIC GARLIC EXTRACT

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

Table No.1: Chemical compounds of major families in Ethanol 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

Gross Fetus Analysis

Acetaminophen acted as toxicant showed lower fetal survival in a dose-dependent manner, with mortality increasing in Group C (APAP at 300mg/kg/B.W) as compared to Group B (APAP at 150mg/kg/B.W). Ethanolic Garlic Extracts (EGE) co-administration increased survival ratio of embryo's across all groups (D-G). EGE dose of 200mg/kg/B.W Improved Survival rates in Groups (E and G), as compared to dose of 100mg/kg/B.W in Groups (D and F) as shown in Table No.2. This is due to bioactive agents in Garlic Extract, particularly amino acid derivative like N-Acetyl Cysteine (NAC), Whereas total recovered embryos from each group is shown in Table No.2

Table No. 2: Total embryos recovered from each group (A-G)

Treatment Groups	Total Fetus Obtained	Fetus Alive	Fetus Dead
Control (A)	38	38	Nil
APAP 150 mg (B)	30	28	2
APAP 300mg (C)	25	20	5
APAP+EGE 150mg+100mg (D)	28	25	3
APAP+EGE 150mg+200mg (E)	30	28	2
APAP+EGE 300mg+100mg (F)	23	20	3
APAP+EGE 300mg+200mg (G)	24	22	2

Where, APAP = Acetaminophen; EGE = Ethanolic Garlic extract

Morphometrical Analysis

Acetaminophen was used as toxicant to pregnant female albino mice and ethanolic garlic extract was used as curative agent in this experiment. All treatments were given via oral gavage to ensure precise dosing. 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 from Morphometric analysis showed that fetus's weight, hind limb, fore limb, tail and snout length, crown rump, and head circumference decreased significantly ($P<0.05$) in Acetaminophen treated groups B and C as compared to control group A as shown in Table No.3. Co-administration of ethanolic garlic extracts (EGE) exhibit overall protective properties in ameliorative groups (D-G). Curative agent (EGE at 200 mg/kg/B.W) in Groups E and G showed significant protective efficacy than (EGE at 100 mg/kg/B.W) in Groups D and F. This is due to biological active compounds present in Garlic extract.

Morphological Observations

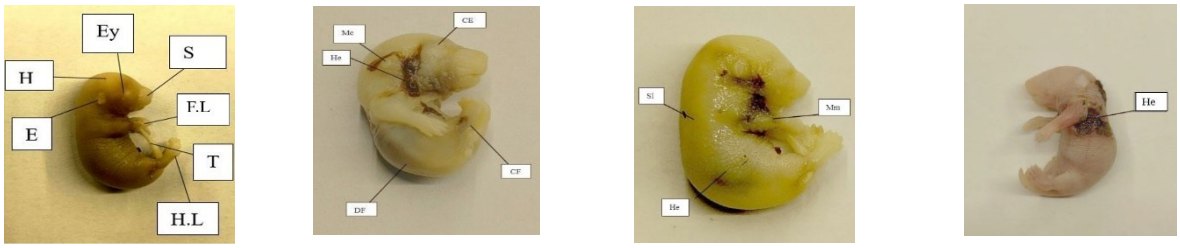
Acetaminophen was currently administered with Ethanolic Garlic extracts (EGE) orally to pregnant mice (Mus Musculus). All treatments were given via oral gavage to ensure precise dosing. The control group received only distilled water and food with regular intervals, it showed no abnormalities as shown in the figure No.2. Acetaminophen as toxicant was used at a dose of 150mg/kg/BW and 300mg/kg/BW, whereas ethanolic garlic extracts (EGE) were simultaneously dosed as an ameliorative agent at a dose of 100 and 200mg/kg/BW. Results exhibited significant developmental abnormalities like: Hemorrhage (He), Deformed axis (DF), Micromelia (Mm), Macrotia (Mc), club feet (CF) and closed eyes (CE) in toxicant (APAP) treatment of 300mg/kg, whereas ameliorant (EGE) co-administration significantly reduced the adverse effects of acetaminophen. Our results recorded a significant protective potential of Ethanolic garlic extracts (EGE) when given at dose level of 200mg/kg/BW as compared to the dose at 100mg/kg/BW. As shown in the figure No.2. which is due to the bioactive compounds present in the garlic extract.

Table No.3 Morphometric parameters of Acetaminophen administration on mice embryo with co-treatment of ethanolic garlic extracts.

Doses groups mg/kg/B.W	Fetus weight (mg)*	Hind limb (mm)*	Fore limb (mm)	Tail Length (mm)	Snout length(mm)	Crown rump length (mm)	Head circumference (mm)
Control (A)	1209.62 ± 0.99[a]	9.35 ± 0.36a	8.83 ± 0.36[a]	12.20 ± 0.27[a]	4.20 ± 0.19[a]	15.40 ± 0.14[a]	20.24 ± 0.71[a]
APAP 150 mg (B)	1111.55 ± 1.04[b]	9.03 ± 0.39b	8.52 ± 0.33[b]	12.17 ± 0.29[b]	4.11 ± 0.16[b]	14.74 ± 1.98[b]	19.85 ± 0.17[b]
APAP 300mg (C)	1094.63 ± 1.03[c]	8.84 ± 0.43[c]	7.63 ± 0.27[c]	10.25 ± 0.23[c]	3.96 ± 0.14[c]	14.98 ± 0.19[b]	19.83 ± 0.20[b]
APAP+EGE 150mg+100mg (D)	1161.54 ± 0.83d	9.17 ± 0.26[d]	8.68 ± 0.23[d]	12.09 ± 0.27[a]	4.05 ± 0.15[d]	15.11 ± 0.16[a]	20.08 ± 0.19[a]
APAP+EGE 150mg+200mg (E)	1181.77 ± 0.76[e]	9.27 ± 0.18[e]	8.79 ± 0.31[b]	12.30 ± 0.26[a]	4.12 ± 0.10[b]	15.32 ± 0.15[a]	20.24 ± 0.14[a]
APAP+EGE 300mg+100mg (F)	1146.70 ± 0.84[f]	9.10 ± 0.26[f]	8.57 ± 0.38[b]	12.17 ± 0.16[a]	4.22 ± 0.16[a]	15.13 ± 0.17[a]	20.23 ± 0.18[a]
APAP+EGE 300mg+200mg (G)	1164.43 ± 0.84[g]	9.30 ± 0.16[a]	8.87 ± 0.44[a]	12.17 ± 0.20[a]	4.29 ± 0.14[a]	15.33 ± 0.16[a]	20.25 ± 0.19[a]

Values bearing the same letters are insignificant and vice versa (P<0.05). Where, APAP = Acetaminophen; EGE = Ethanolic Garlic extract

Figure No.2: Morphological features of mice fetuses recovered after co-treatment of Acetaminophen and Ethanolic Garlic Extract different doses



(Control) **(APAP at 300mg)** **(APAP 300mg+EGE 100mg)** **(APAP 300mg+EGE 200mg)**

Where as, **H = Head; E= Eyers. Ey = Eye; FL = Fore Limb; HL = Hind Limb; S = Snout; T = Tail; DF = Deformed; He = Hemorrhage; OE = Open Eyelid; Mm = Micromelia; Sl = Skin Lesion; CF = Club Feet; Mc=Macrotia.**

Discussion

Acetaminophen is a widely used pain-relieving agent. The study was aimed to examine the acetaminophen (N-acetyl-para-aminophenol) induced embryotoxicity in female albino mice and ameliorative potential of garlic (Allium sativum) extract. For this purpose, Phytochemical analysis of ethanolic garlic extract was performed. 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 like N-Acetyl cysteine have ameliorative potential.

Reduction of fetal Survival rates were recorded in this study across all the Acetaminophen treated groups, similar observations were observed by reports of Burdan et al, 2001 in pregnant wister rats, that showed fetal resorption and implantation failure in embryos, which is also in agreement with work of Kristensen et al., 2024, they studied the lowering of viable fetus when dosed with 200mg/kg of acetaminophen, also finding out that this drug inhibited cell cycle progression during embryogenesis, likely through ribonucleotide reductase, resulted in blockage of DNA synthesis, and reduced progeny in mouse models were seen.

Morphometric Analysis showed that fetus's weight, hind limb, fore limb, tail and snout length, crown rump, and head circumference decreased significantly in Acetaminophen treated groups is in accordance with the studies of Ma et al., 2023, who studied effects of acetaminophen dose (100-400mg/kg) in kunming mice, which confirmed that acetaminophen reduce bone lengths in developing embryos primarily due to interreference of this drug with canonical WNT signaling pathway that governs osteogenic differentiation during embryogenesis, which also accords with Chen Z et al., 2024, they treated pregnant mice with acetaminophen at dose of (100-400g mg/kg) and reported a dose dependent reduction in growth parameters of embryos. Whereas, Fetal weight reduction with increasing acetaminophen is in agreement with the studies of Dai Y et al., 2024, they use variable acetaminophen dose (10-150mg/kg) on pregnant mice and recorded low birth weights in embryos recovered, which also coincides with our findings.

Morphological observations recorded developmental abnormalities in acetaminophen treatment like Hemorrhage, Deformed axis, Macrotia, Micromelia, club feet, skin lesions, open eyelid. which were similar with the findings of Burdan et al., 2009. They recorded prenatal ibuprofen exposure produced external malformations in cultured embryos, glutathione level decreased with increasing toxicant concentration, lead towards formation of reactive oxygen species (ROS), which can lead towards tissue necrosis, which coincides with our findings.

Curative Potential of Garlic (Allium sativum) against Acetaminophen induced embryotoxicity is focus of our study. Ethanolic Garlic Extracts (EGE) were used against acetaminophen as curative agent. Our Findings exhibited lowering of teratogenic effects against acetaminophen administration. Garlic treatment reduced the morphometric and morphological abnormalities of fetus induced by different doses of acetaminophen. Ethanolic Garlic extract (EGE) dose at 200mg/kg/B.W is more effective than 100mg/kg/B.W, which is an agreement with the study of Assayed et al. (2010) who observed reduction in external morphological deformities in rat embryos, dosed with cypermethrin during pregnancy with co-administration of garlic extracts as antidote in his experiment. It also accords with the studies of Jang et al., 2018, that indicates the presence of biologically active compounds, specifically Amino acid derivates like N-Acetyl Cystiene, that act as a precursor molecule for glutathione synthesis, that helps in scavenging of reactive oxygen species (ROS). In elevated levels of ROS, antioxidative stress cause damage in internal organs. N-Acetyl Cysteine (NAC) has the ability to ameliorate the effects of acetaminophen in pregnant albino mice.

Conclusion

Results of this study confirmed the Acetaminophen induced embryotoxicity. Morphological observations showed abnormalities in embryos. Higher level of toxicant cause abnormal fetal morphometrical parameters. 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 maintaining the precursors of oxidative stress like glutathione and MDA. Improvement in overall embryo health 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, Morphology, Histology observation and Morphometrical Analysis [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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