EFFECT OF AQUEOUS LEAVES EXTRACTS OF *LAUNAEA TARAXACIFOLIA* ON CASPASE 3 AND CASPASE 9 GENE EXPRESSIONS IN BENZENE-INDUCED HAEMATOTOXICITY IN ALBINO WISTAR RATS

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ABSTRACT

Background: *Launaea taraxacifolia* is used in various traditional medicines for its health benefits, due to its reported antioxidant, anti-inflammatory and cytoprotective properties. One specific area of interest lies in its potential to modulate apoptotic pathways, as aberrant apoptosis regulation has been implicated some disease states, including those involving benzene-induced haematotoxicity. Central to the execution of apoptosis are Caspase 3 and Caspase 9 which hold important roles in mediating both the intrinsic and extrinsic apoptotic pathways.

Aim: This aim of this study was to determine the effect of aqueous leaf extracts of *Launaea taraxacifolia* on Caspase 3 and Caspase 9 gene expression in benzene induced haematotoxicity in albino Wistar rats.

Methods: A total of sixty adult male rats were divided into six groups namely groups A, B, C, D, E and F, representing control, benzene group, cyclophosphamide group, benzene + 100mg/kg *Launaea taraxacifolia*, benzene + 200mg/kg *Launaea taraxacifolia* and benzene+ 400mg/kg *Launaea taraxacifolia* respectively. Haematological parameters, mRNA caspase 3 and mRNA caspase 9 were determined using haematology autoanalyzer, and polymerase chain reaction respectively. Data obtained was analyzed by the Statistical Package for Social Science (SPSS) software.

Results: Haemoglobin concentration and Haematocrit was significantly lower in group C when compared to other groups ($p<0.05$). The mRNA expression of caspase 3 was significantly lower in group B when compared to group A ($p<0.05$). The mRNA expression of caspase 9 was significantly higher in group B when compared to group A ($p<0.05$). The mRNA expression of caspase 9 in groups D, E and F was significantly lower when compared to group B ($p<0.05$).

Conclusion: This study concludes that benzene exposure induced a reduction in caspase 3 mRNA expression, while caspase 9 mRNA expressions significantly increased. Co-administration of benzene and varying *Launaea taraxacifolia* concentrations attenuated the effects on caspase 3 and caspase 9 expression caused by benzene

Key words: *Launaea taraxacifolia,* Caspase 3 gene, Caspase 9 gene, Haematological parameters, Benzene.

INTRODUCTION

The use of medicinal plants as a source of medicine is an ancient practice that has been followed by various cultures around the world for centuries. Many traditional systems of medicine have relied heavily on medicinal plants for treating various ailments GuribFakim (2006).

Medicinal plants contain a wide range of bioactive compounds, including alkaloids, flavonoids, terpenoids, and phenolic compounds, which can have therapeutic effects on the human body. These compounds can exhibit various pharmacological properties, such as anti-inflammatory, antimicrobial, analgesic, antioxidant, and anticancer activities Bernhoft (2010). *Launaea taraxacifolia*, commonly known as African lettuce or wild lettuce, is a medicinal plant that belongs to the Asteraceae family. It is native to Africa and is found in various regions across the continent, including West Africa Dansi *et al*. (2012). The plant is found singly or in clusters of rocky soil, but it is also cultivated in small open gardens near homes for family consumption Koukoui *et al*. (2017). Caspase-3 is one of the key executioner caspases and is involved in the final stages of apoptosis. It is activated during apoptosis by proteolytic cleavage, which leads to its activation and subsequent cleavage of other proteins in the cell, resulting in the dismantling of cellular components and cell death Araya *et al*. (2021). In cancer, caspase-3 is often down-regulated or inactivated, contributing to the resistance of cancer cells to apoptosis and promoting tumor growth and survival. The Caspase 9 gene is a protease that plays a crucial role in programmed cell death, specifically in the intrinsic apoptotic pathway Kim *et al*. (2015). The intrinsic apoptotic pathway is activated in response to various cellular stresses, such as DNA damage or developmental cues, and involves the release of cytochrome C from the mitochondria into the cytosol. Caspase-9 is then recruited to form a multiprotein complex called the apoptosome, which consists of caspase-9, Apaf-1 (apoptotic protease-activating factor 1), and cytochrome C. Once activated within the apoptosome, caspase-9 cleaves and activates downstream effector caspases, such as caspase-3 and caspase-7, ultimately leading to the execution of apoptosis Pawlowski and Kraft (2000). Benzene is hazardous to the blood and blood-forming and exposure to it can impair various haematological parameters and eventually lead to malignancies of the blood and blood-forming organs Hayes *et al*. (2001).

An understanding the modulation of caspase 3 and caspase 9 gene expressions in benzene induced haematotoxicity is crucial for deciphering some of the mechanisms that regulate apoptosis. Investigating the effect of *Launaea taraxacifolia* on these key caspases can provide important insights into its potential role in modulating apoptotic pathways under induced haematotoxicity, which could have implications for various diseases where apoptosis dysregulation is involved like cancer. The aim of this study therefore, is to determine the effect of *Launaea taraxacifolia* leaf extracts on Caspase 3 and Caspase 9 gene expressions in benzene-induced haematotoxicity in albino Wistar rats.

MATERIALS AND METHODS

Study Population

In this study, animal (rats) model was used. A total of sixty (60) of the Albino Wistar strain were purchased from the animal holdings of the Department of Anatomy, University of Benin, Benin City, Nigeria. The rats were housed at the animal housing wing of the Department of Anatomy, University of Benin Obazelu and Faluyi (2023).

Identification of the *Launaea taraxacifolia* Leaves

Launaea taraxacifolia leaves were harvested from Faculty of Agriculture, University of Benin, Nigeria. The leaves were then identified and authenticated by Dr. A. O Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City Obazelu *et al*. (2021).

Preparation of Plant Extract

About 3.65kg leaves were pulverized (after drying) by a commercial blender and (665g of powder obtained), soaked in distilled water using 1g of powder to 5ml of distilled water and allowed to stand for 72 hours at room temperature.

The extract was filtered using Whatman's (Nitro cellulose 45; 0.45µm pore size) filter paper and the filtrates were concentrated to dryness at 100ºC in a water bath. Thereafter, it was put in an airtight container and refrigerated until use Obazelu *et al*. (2021).

Animal Care

Animals were housed in a cross ventilated room in the animal holdings of the department of anatomy, University of Benin, Benin City. Animals were exposed to 12 hours dark and light cycles with access to feed and water *ad libitum*. The rats were acclimatized for a period of two (2) weeks before commencement of the experiment Obazelu *et al*. (2021).

Ethical Consideration

Ethical approval was obtained from Research Ethics Committee on animal subjects from Edo State Ministry of Health, Benin City (Ref Number: HA/737/23/B/200600143 issued on 30th June, 2023).

Preparation of Benzene and Cyclophosphamide Drug Solution

Benzene Solution

Benzene solution was made by mixing benzene (Manufactured by LOBA Chemie Laboratory Reagents and Fine Chemicals Ltd, Batch Number: L246641711), distilled water v/v and 2-propanol in the ratio of 1:5:5. That is, 1 part of benzene was mixed with 5 part of distilled water v/v and 5 parts of 2-propanol. 0.2ml of this benzene solution was administered to each animal in the various test groups with an average weight of 150g every 48 hours for 28 days Obazelu and Faluyi (2023)

Cyclophosphamide Drug Solution

Cyclophosphamide drug solution was made by mixing 500mg of the powdered drug in 25ml of distilled water. 0.3ml of this drug solution was administered orally to each animal in group C of an average weight of 150g every 48 hours for 28 days Akanni *et al*. (2014).

Research Design

Grouping of Animals: Sixty (60) Mature Wistar rats weighing 150-200g were randomly selected and divided into six groups (n = 10 per group). The Groups were the Group A, Group B, Group C, Group D, Group E and Group F.

Group A: This was the control group. Animals in this group received only standardized feed (Manufactured by Karma Agric. Feeds and Food limited, Oyo State) and clean water *ad libitum.*

Group B: This group received only benzene intraperitoneally.

Group C: Animals in this group were administered benzene solution and treated with the standard drug solution (cyclophosphamide) intraperitoneally.

Group D: Animals in this group were administered benzene solution intraperitoneally and treated with low dose of *Launaea taraxacifolia* leaves extract orally.

Group E: Animals in this group were administered benzene solution intraperitoneally and treated with a higher dose of *Launaea taraxacifolia* leaves extract orally.

Group F: Animals in this group were administered benzene solution intraperitoneally and treated with the highest dose of *Launaea taraxacifolia* leaves extract orally Obazelu and Faluyi (2023).

Administered Doses of *Launaea taraxacifolia* Extract

Group A (control) received only standardized feed and clean water *ad libitum*. Group B (benzene treated group) were administered 0.2ml of benzene solution intraperitoneally every 48 hours for 28 days. Group C (cyclophosphamide drug solution treated group) were administered 0.2ml of benzene solution intraperitoneally every 48 hours for 28 days and treated with 0.3ml of 6mg/ml of cyclophosphamide 48 hourly for 28 days. Group D were administered with 0.2ml of benzene solution intraperitoneally every 48 hours for 28 days and treated with 0.15ml of 100mg/kg body weight of *Launaea taraxacifolia* leaves extract orally using a gavage tube every 24 hours for 28 days.

Group E were administered with 0.2ml of benzene solution intraperitoneally every 48 hours for 28 days and treated with 0.3ml of 200mg/kg body weight of *Launaea taraxacifolia* leaves extract orally using a gavage tube every 24 hours for 28 days. Group F were administered with 0.2ml of benzene solution intraperitoneally every 48 hours for 28 days and treated with 0.6ml of 400mg/kg body weight of *Launaea taraxacifolia* leaves extract orally using a gavage tube every 24 hours for 28 days Obazelu and Faluyi (2023).

Sacrifice of Animals and Collection of Samples

At the end of the experimental period, the animals were grossly observed for general physical characteristics. A midline incision was made through the ventral wall of the rats after anaesthetizing (using chloroform) and cervical dislocation. Five milliliters (5ml) of blood were collected from each rat using a sterile syringe and placed in an Ethylene Diamine Tetra-acetic Acid (EDTA) container for full blood count analysis and peripheral blood film preparation. Bone marrow samples were also obtained from the rats by opening the femur longitudinally and exposing the marrow cavity. A sterile forceps was used to obtain the bone marrow from the cavity and placed in an Eppendorf container containing Trizol for molecular analysis Obazelu and Faluyi (2023).

Haematological Profile Analysis

The full blood count parameters were analysed immediately after sample collection using the automated three parts ERMA Haematology Auto analyser PCE-210N (Diamond Diagnostic; Holliston, USA). Calibration and standardization of the equipment, processing and analysis of the samples were done strictly according to the manufacturer's instructions.

Procedure

The whole blood was properly mixed and inserted into the probe. Then 20 µL of the blood was aspirated into the instrument. The analysis was immediately done and the results displayed on the screen after about 1-2 minutes, which was printed by the printer.

Caspase 3 and Caspase 9 mRNA Assay

Isolation of Total RNA

Total RNA was isolated from tissue samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

cDNA conversion

One (1 µg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min Elekofehinti *et al*. (2020).

PCR Amplification and Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) for the amplification of caspase 3 and caspase 9 genes was carried out with OneTaqR2X Master Mix (NEB) using the following primers (Inqaba Biotec, Hatfield, South Africa). PCR amplification was performed in a total of 25µl volume reaction mixture containing cDNA, primer (forward and reverse SEE BELOW) and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95ºC for 5 min, followed by 30 cycles of amplification (denaturation at 95ºC for 30 seconds, annealing for 30 seconds and extension at 72ºC for 60 seconds) and ending with final extension at 72ºC for 10 min. The amplicons were resolved on 1.0% agarose gel. The Glyceraldehyde-3-Phosphate

Dehydrogenase (GAPDH) gene was used to normalize the relative level of expression of each gene, and quantification of band intensity was done using "image J" software Elekofehinti *et al*. (2020).

SYNTHESIZED PRIMER SEQUENCES

Caspase 3

Forward:
TGTATGCTTACTCTACCGCACCCG
Reverse:
TGTATGCTTACTCTACCGCACCCG

Caspase 9

Forward: GCAATCCGCTAGCCATGGAG
Reverse: GAGCCTGCCCGCTGAATA

GAPDH

CTCCCTGGAGAAGAGCTATGA
AGGAAGGAAGGCTGGAAGA

Statistical Analysis

Data obtained from this research was presented and analyzed using statistical package for social sciences (SPSS) version 21.0 (IBM Inc. USA). Analysis of variance (ANOVA) was used to compare treatment groups of continuous variables. Tukey HSD *post hoc* was applied where a significant difference was observed in the ANOVA. Bar chart was used to represent the mRNA gene expression patterns. A p value of ≤0.05 was considered statistically significant.

RESULTS

Table 1 shows the comparison of Mean±SD of red blood cell count, Haemoglobin concentration, haematocrit and red cell indices of six groups namely; groups A, B, C, D, E and F, representing control, benzene group, cyclophosphamide group, benzene + 100mg/kg *Launaea taraxacifolia*, benzene + 200mg/kg *Launaea taraxacifolia* and benzene + 400mg/kg *Launaea taraxacifolia* respectively.

Red blood cell count (RBC) (µL) of groups A (9.3±0.08), B (9.376±0.13), D (9.045±0.12), E (8.894±0.05) and F (8.939±0.03) was significantly higher when compared to group C (7.768±0.11) ($p<0.05$). Haemoglobin concentration (g/dL) was significantly higher in groups A (14.23±0.33), B (14.51±0.08), D (14.76±0.34), E (14.3±0.15) and F (14.57±0.06) when compared to group C (12.62±0.12) ($p<0.05$). Haematocrit (%) was significantly higher in groups A (42.59±1.01), B (43.95±0.30), D (43.54±1.03), E (42.9±0.50) and F (43.67±0.38) when compared to group C (37.66±0.42) ($p<0.05$). Mean cell volume (MCV) (μm^3) was significantly lower in group A (45.81±0.72) when compared to groups C (48.49±0.30), D (48.06±0.51), E (48.23±0.48) and F (48.84±0.28) ($p<0.05$). Mean cell haemoglobin (MCH) (pg) was significantly lower in group A (15.3±0.24) when compared to groups C (16.27±0.16), D (16.27±0.16), E (16.08±0.11) and F (16.32±0.05) ($p<0.05$). Mean cell haemoglobin concentration (MCHC) (g/dL) was significantly lower in group B (33±0.24) when compared to groups C (33.68±0.04) and D (33.9±0.09) ($p<0.05$). RDWS (μm^3) of group A (24.41±0.02) and Group E (24.3±0) was significantly lower when compared to group C (26.24±0.4) ($p<0.05$).

Table 1. Mean Comparison of Red Blood Cell Indices of Studied Groups

Parameters	Group A (Control) (n=10)	Group B (Benzene only) (n=10)	Group C (Benzene + Cyclophosphamide) (n=10)	Group D (Benzene + 100mg/kg) (n=10)	Group E (Benzene + 200mg/kg) (n=10)	Group F (Benzene + 400mg/kg) (n=10)	F value	p value
RBC ($\times10^6/\mu L$)	9.3 ± 0.08^{ce}	9.376 ± 0.13^{cef}	7.768 ± 0.11^{abdef}	9.045 ± 0.12^{c}	8.894 ± 0.05^{abc}	8.939 ± 0.03^{bc}	35.78	<0.0001
Haemoglobin (g/dL)	14.23 ± 0.33^{c}	14.51 ± 0.08^{c}	12.62 ± 0.12^{abdef}	14.76 ± 0.34^{c}	14.3 ± 0.15^{c}	14.57 ± 0.06^{c}	12.94	<0.0001
HCT (%)	42.59 ± 1.01^{c}	43.95 ± 0.30^{c}	37.66 ± 0.42^{abdef}	43.54 ± 1.03^{c}	42.9 ± 0.50^{c}	43.67 ± 0.38^{c}	12.06	<0.0001
MCV (fl)	45.81 ± 0.72^{cdef}	46.95±0.44	$48.49\pm0\text{-}.30^{a}$	48.06 ± 0.51^{a}	48.23 ± 0.48^{a}	48.84 ± 0.28^{a}	5.55	0.0003
MCH (pg)	15.3 ± 0.24^{cdef}	15.5 ± 0.16^{cdf}	16.27 ± 0.16^{ab}	16.27 ± 0.16^{ab}	16.08 ± 0.11^{a}	16.32 ± 0.05^{ab}	7.347	<0.0001
MCHC (g/dL)	33.43±0.03	33 ± 0.24^{cd}	33.68 ± 0.04^{b}	33.9 ± 0.09^{b}	33.34±0.18	33.36±0.14	4.515	0.0016
RDWC (%)	15.04±0.22	15.34±0.29	15.86±0.06	16.15±0.59	15.16±0.35	16.03±0.09	2.166	0.0714
RDWS (μm^3)	24.41 ± 0.02^{c}	24.78±0.2	26.24 ± 0.4^{ae}	25.56±0.68	24.3 ± 0^{c}	25.05±0.28	3.987	0.0038

Key: $p\leq0.05$- Significant; $p \geq 0.05$- Not significant. a represents significance with control, b represents significance with benzene group, c represents significance with benzene + cyclophosphamide group, d represents significance with benzene + 100mg/kg, e represents significance with benzene + 200mg/kg, f represents significance with benzene + 400mg/kg.

RBC: Red Blood Cell, HCT: Haematocrit, MCV: Mean Cell Volume, MCH: Mean Cell Haemoglobin, MCHC: Mean Cell Haemoglobin Concentration, RDWC: Red cell distribution width – Coefficient of Variation, RDWS: Red cell distribution width – Standard Deviation

Figure 1 shows the expression of genes as represented by gel electrophoresis picture and internal control (Glycealdehyde-3-Phosphate Dehydrogenase {GADPH}) of mRNA expression of Caspase 3 of groups A, B, C, D, E and F, representing control, benzene group, cyclophosphamide group, benzene + 100mg/kg *Launaea taraxacifolia*, benzene + 200mg/kg *Launaea taraxacifolia* and benzene + 400mg/kg *Launaea taraxacifolia* respectively, represented on different bars on the bar chart. There was a significant decrease in the mRNA expression of caspase 3 of group B when compared to group A ($p<0.05$). Groups C, D, E and F showed statistically significant higher expression of caspase 3 when compared to group B ($p<0.05$).

Figure 2 shows the expression of genes as represented by gel electrophoresis picture and internal control (Glycealdehyde-3-Phosphate Dehydrogenase {GADPH}) of mRNA expression of Caspase 9 of groups A, B, C, D, E and F, representing control, benzene group, cyclophosphamide group, benzene + 100mg/kg *Launaea taraxacifolia*, benzene + 200mg/kg *Launaea taraxacifolia* and benzene + 400mg/kg *Launaea taraxacifolia* respectively, represented on different bars on the bar chart. There was a significant increase in the mRNA expression of caspase 9 of group B when compared to group A ($p<0.05$). Groups D, E and F showed statistically significant lower expressions of caspase 9 when compared to group B ($p<0.05$).

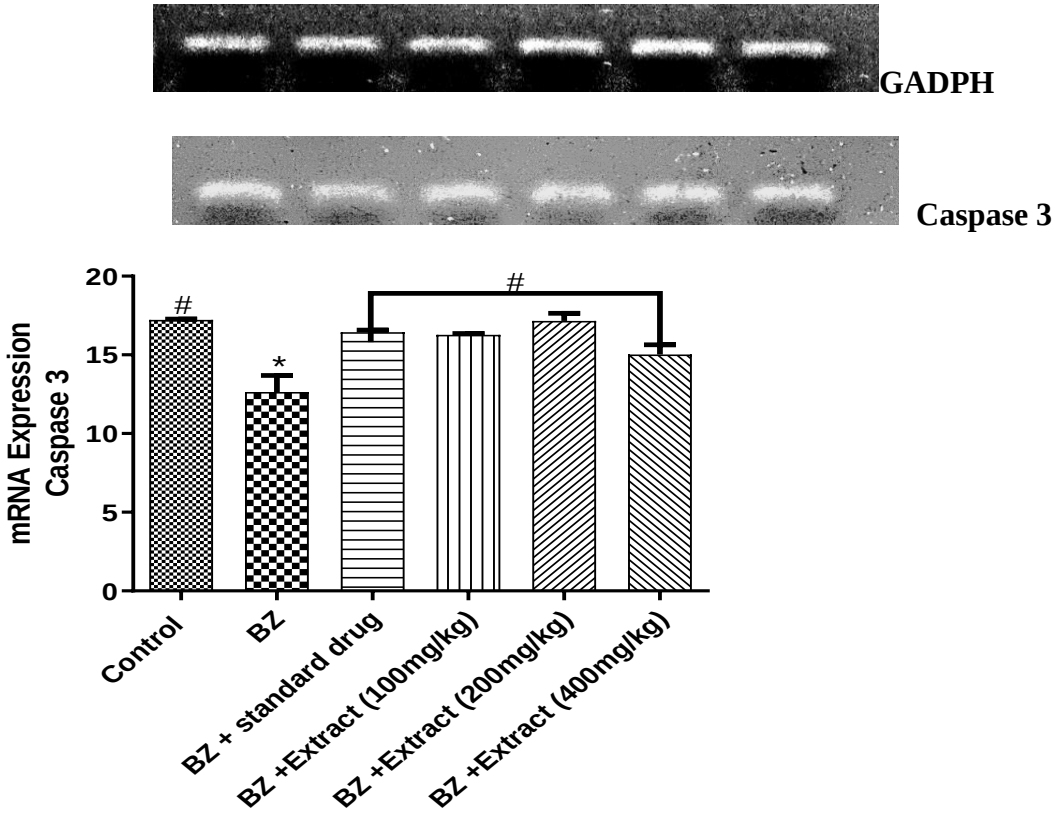

Figure 1. mRNA Expression of Caspase 3 of the Studied Groups.
* Represents statistical difference to control. # Represents statistical difference to benzene induced group at p<0.05. Key: BZ=Benzene, GADPH=Glyceraldehyde-3-Phosphate Dehydrogenase

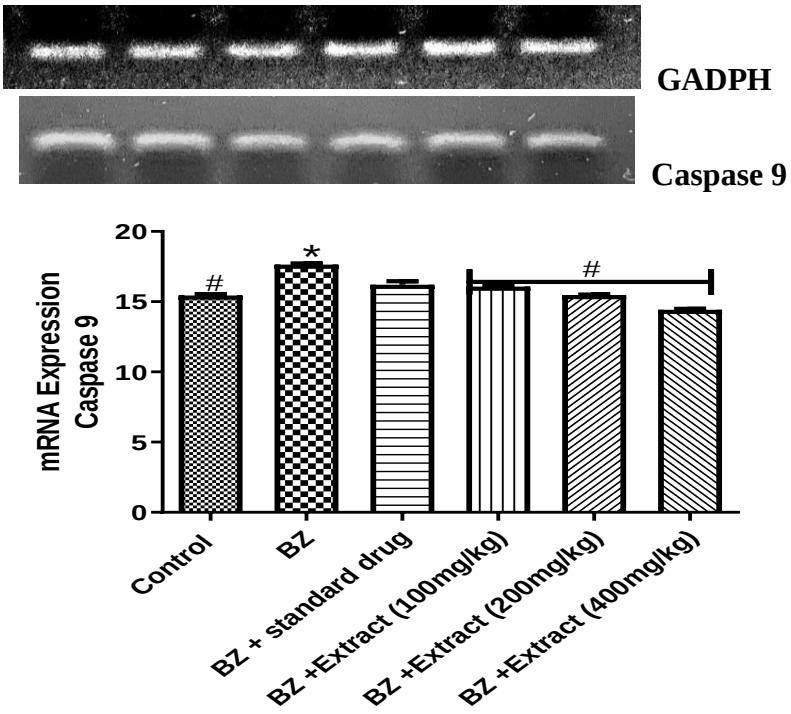

Figure 2. mRNA Expression of Caspase 9 of the Studied Groups.
*Represents statistical difference to control. # Represents statistical difference to benzene induced group at p<0.05. Key: BZ=Benzene, GADPH=Glyceraldehyde-3-Phosphate Dehydrogenase

DISCUSSION

This study revealed that benzene administration did not induce any significant alterations in red blood cell parameters, including red blood cell count, Haemoglobin concentration, hematocrit levels which are in agreement with a study carried out by Saha *et al*. (2012). Administration of cyclophosphamide however, resulted in reduction in red blood cells, haemoglobin and haematocrit. This result disagrees with that of Ukpo *et al*. (2013) who reported no significant change in these parameters after administration of cyclophosphamide to male albino Wistar rats. Cyclophosphamide-induced reductions in red blood cell count, Haemoglobin levels, and hematocrit levels are consistent with its cytotoxic effects on erythropoiesis. Also, the observed increase in mean corpuscular volume (MCV), mean corpuscular Haemoglobin (MCH), mean corpuscular Haemoglobin concentration (MCHC), and red cell distribution width (RDW-SD) following cyclophosphamide administration highlights the potential for disruptions in erythrocyte size and Haemoglobin content due to the cytotoxic impact of the compound. This result was also not consistent with that of Ukpo *et al*. (2013) who recorded no significant change in red cell indices due to cyclophosphamide. The administration of 100mg/kg, 200mg/kg, and 400mg/kg doses of *Launaea taraxacifolia* with benzene, led to a significant increase in MCV, MCH, and MCHC. These red cell indices reflect erythrocyte size, Haemoglobin content per cell, and Haemoglobin concentration per unit volume, respectively. The observed increase in these indices might suggest the potential of *Launaea taraxacifolia* to influence erythrocyte maturation and Haemoglobin synthesis, however further investigation might be needed to decipher the precise molecular mechanisms responsible for these effects.

Caspase 3 is often referred to as an "executioner" caspase because of its role in carrying out the biochemical events that lead to cell death during apoptosis Grutter (2000). In this study, there was a significant reduction in the mRNA expression of Caspase 3 following benzene exposure. Given that Caspase 3 is a pivotal player in the execution of apoptosis, its reduced expression might indicate a potential compensatory mechanism aimed at reducing apoptosis in response to cellular stress induced by benzene administration. This finding is however in contrast with that of Wen *et al*. (2016) who observed an increase in Caspase 3 expression due to benzene administration. Co-administration of benzene with varying concentrations of *Launaea taraxacifolia* did not result in a significant reduction in Caspase 3 mRNA expression, despite a slightly lower expression level observed in the 400mg/kg concentration group. This implies that *Launaea taraxacifolia* might have the potential to modulate the impact of benzene on Caspase 3 gene expression, potentially attenuating the suppressive effects induced by benzene. Caspase 9 is a central "initiator" caspase that plays a crucial role in initiating the intrinsic apoptotic pathway in response to various cellular stressors, such as DNA damage, oxidative stress, or cellular damage Radi *et al*. (2014). This study revealed a significant increase in the mRNA expression of Caspase 9 following benzene exposure. This elevation in Caspase 9 gene expression suggests the activation of apoptotic pathways in response to the toxic effects of benzene exposure. A study carried out by Bahadar *et al*. (2015) also reported an increase in expression of caspase 9 after benzene administration. Co-administration of benzene with varying concentrations of *Launaea taraxacifolia* did not result in a significant increase in Caspase 9 mRNA expression. Although the expression levels of Caspase 9 were slightly lower in the 400mg/kg concentration group, the lack of a significant increase suggests a potential modulatory effect of *Launaea taraxacifolia* on Caspase 9 gene expression in the presence of benzene.

This modulation might indicate a regulatory influence of *Launaea taraxacifolia* on the apoptotic pathways activated by benzene, potentially attenuating the upregulation of Caspase 9 as a response to cellular stress.

CONCLUSION

Data from this study showed that benzene exposure induced a reduction in caspase 3 mRNA expression, while caspase 9 mRNA expressions significantly increased. Co-administration of benzene and varying *Launaea taraxacifolia* concentrations attenuated the effects on caspase 3 and caspase 9 expression caused by benzene.

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