CYTOPROTECTION BY LEAF EXTRACT OF *Tridax procumbens* (LINN.) IN EMBRYONIC MOUSE, HEPG2 AND PANC-1 CELL LINES AND ARSENITE-INDUCED TOXICITY IN RATS

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CERTIFICATION

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DEDICATION

I wholeheartedly dedicate this research project to the almighty God. I also dedicate this project to all cancer patients who contributed to my motivation for enrolling in the Ph.D. programme. Also to my hardworking Parents, who gave me an educational foundation. And lastly to my beloved Wife and Children who showed love, understanding, and supported me throughout the period of this Ph.D. programme.

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDA) and Hepatocellular Carcinoma (HCC) are among the deadliest cancer types worldwide that are associated with arsenic intoxication. Current drugs used in cancer management show a lot of side effects. There is, therefore, an increased search for alternatives from medicinal plants with anticancer properties. *Tridax procumbens* (TP) is a medicinal plant rich in antioxidant phytochemicals with little information on its effects on cancer and arsenic toxicity. This study was designed to investigate the effect of *Tridax procumbens* in PDA, HCC and arsenite-induced toxicity using *in vivo* and *in vitro* models.

The TP was authenticated (UIH-22542), air-dried, blended, soaked in ethanol, and concentrated to obtain the Crude Extract (CETP). The CETP was fractionated using Hexane, Dichloromethane, and Ethyl Acetate to obtain Fractions (HXF, DCMF, and EAF, respectively). Antioxidant assays of TP were performed. For *in vivo* study, 32 male Wistar rats (80-100g) were assigned into four groups (n=8), and treated as follows; A (control); 1 ml/kg body weight olive oil, B; 2.5 mg/kg Sodium Arsenite (SA), C; 50 mg/kg CETP, D; SA+CETP. Olive oil and CETP were administered once daily for 14 days, and SA twice (days 7 and 14). Histological examination of lungs and brain, and micronucleated Polychromatic Erythrocytes (mPCEs) frequency were carried out. The HCC and PDA cell lines; HepG2 and Panc-1, were maintained in a humidified incubator, and treated with (10, 20, 50, 100, and 250 μg/ml) dimethyl sulfoxide (control), CETP and CETP-fractions for 24 and 48 hours. Similarly, embryonic mouse pancreas (E11.5d) were cultured for five days and treated with the test samples (20 μg/ml) for two days. Cytotoxicity assays [3-(4,5-dimethylyhiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Live/Dead] were carried out, and expression of proteins (cytokeratin-7, peanut agglutinin (PNA), α-fetoprotein, insulin, glucagon, amylase, catalase, alkaline phosphatase, Glutathione S-transferase-pi (GST-pi), caspase-3, Bcl-2, Adenomatous Polyposis Coli (APC), p53, $p21^{Cip1/Wap-1}$, vimentin, Ki-67, Sox9, β-catenin, α1-antitrypsin, albumin, transferrin) were evaluated using immunofluorescence. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The DCMF scavenged 2,2-diphenyl-1-picrylhydrazyl hydrate and nitric oxide radicals (IC_{50}: 0.43 and 0.39 μg/ml, respectively) relative to control (IC_{50}: 0.85 and 0.14 μg/ml, respectively). The CETP reduced arsenite-induced histological lesions in lungs and brain, and mPCEs [SA (21.00±3.33) to SA+CETP (3.80±0.58)] compared to control (0.60±0.40). The DCMF elicited cell death (IC_{50}=23.1 μg/ml) compared to CETP (IC_{50}=114.2 μg/ml). Relative to control, phenotype morphogenesis was observed in DCMF-treated embryonic pancreas with immunopositivity for insulin, vimentin and amylase (1.3-fold), cytokeratin-7 (1.5-fold), PNA (1.9-fold), and glucagon (2.8-fold). There were significant elevations in transferrin (1.5-fold), albumin (1.8-fold), p53 (2.8-fold), caspase-3 (2.9-fold), catalase (4.0-fold), $p21^{Cip1/Wap-1}$ (4.4-fold), and alkaline phosphatase (5.0-fold) in DCMF-treated cells compared to control. Conversely, there were significant reductions in β-catenin (1.3-fold), α1-antitrypsin and cytokeratin-7 (2.0-fold), PNA and GST-pi (2.3-fold), α-fetoprotein and Ki-67 (2.7-fold), and vimentin (10.8-fold) in DCMF-treated cells relative to control. The DCMF induced Bcl-2 punctate nuclear staining, and cytoplasmic translocation of APC, Sox9, and β-catenin in treated cells.

Leaf extract of *Tridax procumbens* exhibited antiproliferative activities via the decrease in biomarkers of cancer induction and increase cellular antioxidant defence system.

Keywords: *Tridax procumbens*, Arsenite toxicity, Phytochemicals, Phenotype morphogenesis

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ABBREVIATIONS

AAE	-	Ascorbic acid equivalent
AAT	-	Alpha-1-antitrypsin
ACUREC	-	Animal care use and research ethics committee
AFP	-	Alpha-fetoprotein
ALP	-	Alkaline phosphatase
ALT	-	Alanine aminotransferase
ANOVA	-	One-way analysis of variance
APC	-	Adenomatous Polyposis Coli
AQF	-	Aqueous fraction
AST	-	Aspartate aminotransferase
BCL-2	-	B-cell lymphoma 2
BME	-	Basal Medium Eagle
BUN	-	Blood urea nitrogen
BWT	-	Body weight
CAII	-	Carbonic anhydrase II
CETP	-	Crude extract of *Tridax procumbens*
CK7	-	Cytokeratin 7
CREAT	-	Creatinine
DAPI	-	4′,6-diamidino-2-phenylindole
DCMF	-	Dichloromethane fraction
DMEM	-	Dulbecco's Modified Eagle's medium

DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DPPH	-	2,2-diphenyl-1-picrylhydrazyl hydrate
EAF	-	Ethyl acetate fraction
ECACC	-	European collection of authenticated cell cultures
EGFR	-	Epidermal growth factor receptor
EMT	-	Epithelial-mesenchymal transition
FAO	-	Food and Agriculture Organisation
FBS	-	Foetal bovine serum
FRAP	-	Ferric reducing antioxidant power
GAE	-	Gallic acid equivalent
GGT	-	Gamma glutamyl transferase
GLOBOCAN	-	Global Cancer Incidence, Mortality, and Prevalence
GSH	-	Reduced glutathione
GSK-3β	-	Glycogen synthase kinase-3β
GST-pi	-	Glutathione *S*-transferases pi
HB	-	Blood haemoglobin concentration
HDL	-	High density lipoprotein
HPLC	-	High performance liquid chromatography
HXF	-	Hexane fraction
KEAP1	-	Kelch-like ECH-associated protein 1
LC-MS	-	Liquid chromatography - mass spectrometry
LDL	-	Low density lipoprotein

LSM	-	Laser scanning microscope
M/Z	-	Mass-to-charge ratios
MCH	-	Mean haemoglobin concentration
MCHC	-	Mean corpuscular haemoglobin concentration
MCV	-	Mean corpuscular volume
MEMFA	-	MOPS/EGTA/magnesium sulfate/formaldehyde
mPCEs	-	Micronucleated polychromatic erythrocytes
MS	-	Mass spectrometry
MSMS	-	Mass spectrometry - Mass spectrometry
MTT	-	3-(4,5-dimethylyhiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO	-	Nitric oxide
NRC	-	National Research Council
Nrf2	-	Nuclear factor erythroid 2-related factor 2
OECD	-	Organisation for Economic Co-operation and Development
PBS	-	Phosphate buffer saline
PCV	-	Packed cell volume
PFA	-	Paraformaldehyde
PLT	-	Platelet
PNA	-	Peanut agglutinin
QTOF	-	Quadrupole time-of-flight
RBC	-	Red blood cell
REL	-	Relative
RNA	-	Ribonucleic acid

ROS	-	Reactive oxygen species
SA	-	Sodium arsenite
SIPS	-	Stress-induced premature senescence
SLS	-	Scientific Laboratory Supplies Ltd
SOD	-	Superoxide dismutase
TF	-	Thomsen-Friedenreich
TLC	-	Thin layer chromatography
TP	-	*Tridax procumbens*
TRIG	-	Triglyceride
UHPLC	-	Ultra-high performance liquid chromatography
UNECE	-	United Nations Economic Commission for Europe
VLDL	-	Very low density lipoprotein
WBC	-	White blood cell count
WHO	-	World Health Organisation
WNT	-	Wingless-Int
XPC	-	Xeroderma pigmentosum complementation group C

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Plants and their constituents are safe and valuable sources of medicines employed in the management of several disease conditions, hence the term herbal medicine. Acharya *et al.* (2008) described herbal medicine as the use of herbs for meeting both medicinal and therapeutic needs especially in developing countries (Awe and Omojalasola, 2003; Roopan *et al.,* 2019). Approximately three-quarters of the human population does depend on plants to meet health care needs (WHO, 1991).

However, Leonardo *et al.* (2000) further buttress this claim that this is due to the usefulness and reduced side effects of constituents in the herbs. Several drug classes used are prototypes from medicinal plants (Gilani, 2005). For decades now, much attention has been placed on products obtained from plants, especially the medicinal ones (Solecki and Shanidar, 1975; Shu, 1998). Approximately twenty-five per cent of all drugs are obtained from plants (Craig *et al*, 1997).

Therefore, a medicinal plant refers to a plant in which any of its parts comprise substances or compounds that are useful for the production of important products (Sofowora, 1982). The use of the medicinal plant by man is as old as the world herself. In an attempt to remain healthy and to prevent or cure all forms of disease conditions in the body, humans have from time immemorial produced medicine from different parts of plants such as seeds, roots, and leaves (Sofowora, 1982). There are medicinal plants that have received worldwide attention in their use against several conditions (Dalziel, 1956; Turk *et al.*, 2018).

Regrettably, most herbs are employed arbitrarily without assessing their adverse effects. Scientists have documented adverse effects linked with the utilisation of medicinal herbs (Fonseca *et al.*, 1994; Elvin-Lewis, 2001). These adverse effects

include vertigo, nausea, vomiting, abortion, diarrhea, pain, increased heart rate, ulcer, anorexia, and death among others (Gessler, 1995). These effects are ascribed to the existence of phytotoxic components in them and the administration of inaccurate dosage necessary against such diseases (Azaizeh *et al.*, 2003).

To minimise these adverse effects and also make use of the beneficial parts, there is a need for detailed scientific research into the toxicological properties of plants even at varying dosages. The World Health Organisation recommended that native herbs utilised in the management of diseases should be assessed for possible toxicological effects (WHO, 1980).

1.2 Statements of Problem

Cancer is a class of diseases with the features of uncontrolled growth or proliferation of abnormal cells. This class of diseases is supposedly caused mainly by genetic and environmental factors. Statistics revealed that cancer is projected to be named the major reason for mortality and the sole most influential obstacle to improving life expectancy globally (WHO, 2018). Relatively, Ferlay *et al.* (2015) stated that liver cancer is the 2nd between two commonest causes of death resulting from cancer globally. There was a 626,162 incidence of liver cancer in 2002 and the figure rose to 782,451 in the year 2012. Besides, this figure was said to increase by seventy percent to 1,341,344 cases by the year 2035 (Ferlay *et al.*, 2015).

Another deadliest type of cancer is pancreatic cancer especially pancreatic ductal adenocarcinoma having a five-year survival rate of approximately below five per cent (Siegel *et al.*, 2016). Siegel *et al.* (2015) stated that pancreatic cancer has a median time of survival of fewer than six months and an overall survival time of fewer than two years. This is because of its advanced stage presentation, high malignancy, aggressive local invasiveness, and early distal metastasis. These could be the reasons for the poor response of this cancer type to conventional interventions like chemotherapy and radiotherapy. Meanwhile, GLOBOCAN (2016) reported the incidence of all cancer types in Nigeria to be 500,000 for the year 2015.

Hubaux *et al.* (2013) documented that this deadly plaque could arise as a result of atmospheric air, foods, and drinking water pollution, especially in Africa and Asia by

contaminants like arsenic. Arsenicals are environmental pollutants associated with varying consequences after exposure (Liu *et al.*, 2001, Kainth, 2015). The inorganic form of arsenicals predominates in contaminated drinking water that the subjects are exposed to (Bates *et al.*, 1992; Bode and Dong, 2002). Arsenic has posed major environmental challenges because it has been utilised as a component of food preservatives, a byproduct of used fossil fuels, herbicides, and insecticides. And children that are fed on secreted milk from animals that feed on arsenic-contaminated fodder and water are more prone to arsenic toxicity (Brahman *et al.*, 2016).

Several adverse effects in form of toxicity and cancer are all noticed with arsenic exposure (NRC, 1999; Jana *et al.*, 2020). This metalloid is a cancer-causing agent in humans, causing lumps in varying body tissues like the lungs, kidney, liver, skin, and bladder (NRC, 1999, 2014; Liu *et al.*, 2020). Sodium arsenite, a derivative of arsenic has gained scientific interest due to its ubiquitous nature and toxicity (Chatterjee *et al.*, 1995; Samuel *et al.*, 2017; Bhadauria, 2019).

1.3 Study Justification

The widespread environmental contamination by arsenic and the incessant rise in global cancer incidence has increased the search for better preventive and treatment interventions. However, Sofowora, (1982) and Acharya *et al.* (2008) reported the usefulness of medicinal plants as one of the alternative therapies. One of such plants with potential therapeutic efficacy is *Tridax procumbens* L. (Asteraceae). *Tridax procumbens* is naturalised in tropical America and has thrived in other regions of the world (Salahdeen *et al.*, 2004).

Tridax procumbens herb is procumbent and has been employed in the management of wounds, malaria, dysentery, high blood pressure, stomach ache, bronchial catarrh, haemorrhage from cuts and bruises, hair loss, and as feed in animals that could be prone to arsenic exposure (Ali, 2001; Rathi *et al.*, 2008; Talekar *et al.*, 2017; Ara and Islam, 2020). Humans that eat contaminated animals are likely to manifest these toxicities and possibly harbour arsenic-associated cancers in body tissues like lungs, skin, kidneys, pancreas, liver, prostate, and bladder (NRC, 1999, 2014; Waalkes *et al.*, 2003).

Tridax procumbens has insecticidal, antiseptic, parasiticidal, anti-diabetic, antibacterial, anti-inflammatory, analgesic, immune-modulatory, antioxidant and anti-hepatotoxic properties (Pathak *et al.*, 1991; Bhagwat, 2008; Yusuf *et al.*, 2019; Ara and Islam, 2020). Meanwhile, these properties are due to its possession of potent phytochemicals like alkaloids, flavonoids, quercetin, carotenoids, beta-sitosterol, fumaric acid, saponin, anthraquinone, and tannin (Singh and Ahirvar, 2010). However, the lack of information on the effect of *Tridax procumbens* in pancreatic ductal adenocarcinoma (PDA), hepatocellular carcinoma (HCC) and arsenite-induced toxicity using *in vivo* and *in vitro* models warranted this study.

1.4 Research Questions

- Can sodium arsenite induce toxicity in targeted experimental animal tissues?
- Can *Tridax procumbens* extract ameliorate arsenite-induced toxicity?
- Is *Tridax procumbens* extracts cytotoxic to pancreatic ductal adenocarcinoma and hepatocellular carcinoma model cell lines (Panc-1 and HepG2, respectively)?
- Is *Tridax procumbens* extracts toxic to embryonic mouse tissue?
- What is *Tridax procumbens* mechanism of action in Panc-1 and HepG2 cells?
- What bioactive compounds can be responsible for *Tridax procumbens* activities?

1.5 Hypotheses

The study was designed to test the following null hypotheses:

- *Tridax procumbens* extract will ameliorate arsenite-induced dysfunctions in rats.
- *Tridax procumbens* extracts will be cytotoxic to Panc-1 and HepG2 cells.
- *Tridax procumbens* extracts will not be toxic to embryonic mouse tissues.
- Purification will enhance *Tridax procumbens* activities.

1.6 Broad Objective

The research was undertaken to investigate the effect of *Tridax procumbens* in pancreatic ductal adenocarcinoma (PDA), hepatocellular carcinoma (HCC) and arsenite-induced toxicity using *in vivo* and *in vitro* models.

1.6.1 Specific Objectives

- Determine the phytochemicals and antioxidant activity of *Tridax procumbens* extract (CETP) and fractions.
- Assess the possible effects of CETP in Wistar rats exposed to arsenite
- Assess the cytotoxic effects of CETP and its fractions on Panc-1 and HepG2 cells.
- Monitor the effect of CETP and its fractions on embryonic mouse tissues (pancreas, liver, intestine, and oesophagus).
- Assess the effect of CETP most potent fraction(s) on oxidative stress, apoptosis, cell proliferation, differentiation, and metastasis.
- Isolation, purification, and identification of bioactive/unknown compounds from CETP most potent fraction(s) using chromatographic and spectrometric techniques (TLC, column, HPLC, MS, Tandem MS).

CHAPTER TWO

LITERATURE REVIEW

2.1 Cancer

Malignant neoplasm is a class of diseases with the features of unrestrained growth, proliferation, and invasiveness of abnormal cells. If this metastasis or migration is not monitored, it could lead to mortality. The reason many cancers develop is not clear, especially those that affect children. Moreover, there are many reported known risk factors of cancer; modifiable factors like tobacco use, lifestyle, alcohol intake, obesity, and non-modifiable factors including age, sex, hormones, race, and inherited genetic mutations (Siegel *et al.*, 2018). It is pertinent to note that these cancer-associated factors can occur concurrently or in sequence to trigger and enhance the growth and development of malignancy.

Therefore, cancer is supposedly caused mainly by genetic and environmental factors. Non-communicable diseases including cancer have been implicated in several global deaths (WHO, 2018), and cancer is said to be named the major reason for mortality and the sole most influential obstacle to improving life expectancy globally (WHO, 2018). WHO in 2015 reported that 91 out of 172 countries in the world have cancer as either the 1st or 2nd championing cause of mortality before age 70, while in another 22 nations, it was ranked either 3rd or 4th (Bray *et al.,* 2018).

2.1.1 Cancer distribution in Nigeria

Cancer was implicated as the seventh championing reason for mortality in Africa with 1.28 million new cases in a yearly projection and the death of 970,000 by the year 2030 (Ferlay *et al.*, 2014). Factors like population growth, ageing, and elevation in the incidence of other cancer risk associated with an economic transition like poor diet, smoking, physical inactivity, obesity, and reproductive factors make cancer becoming a public health problem in Africa (Jemal *et al.*, 2012). This could also result from poor government policy about the disease (Akinde *et al.*, 2015). Sadly, the World Health

Organisation reported that in Nigeria, more than 71,000 persons died from cancer-associated death and about 102,000 incidences occurred in the year 2012 (Ferlay *et al.,* 2012). While it was reported that in advanced countries such as the USA, both rates of incidence and cancer-related death are reducing, it was the contrary in less advanced countries like Nigeria (Jemal *et al.,* 2010). GLOBOCAN attributed this nearly 102,000 new cases to breast cancer {27,304 (26.7%)}, cervical cancer {14,089 (13.8%)}, liver cancer {12,047 (11.8%)}, and prostate cancer {11,944 (11.7%)} (Ferlay *et al.*, 2012).

The Nigeria National System of Cancer Registries reported 4,209 cancer cases from two cancer registries in Lagos State (2009 - 2013). 25.9% and 74.1% of the data collected were for males and females, respectively. 3,282 cases of cancer were also reported in the Enugu cancer registry with both males and females accounting for 40% and 60% of cases, respectively. Also, Anambra and Edo State recorded cancer cases of 2,024 and 2,230 for males and females, respectively. The cases reported in both Bayelsa and Kogi States were 140 and 187 accordingly (Way and Garki, 2016). A similar trend was observed for all the registries with prostate and breast cancer accounting for the highest common cancer for males and females, respectively.

However, to support this claim, in ten years, it was noticed that the Kano State cancer registry had a progressive rise in cancer cases (Mohammed *et al.,* 2008) and this pattern is in line with the projection of WHO for developing countries (WHO, 2005). Unfortunately, the reported incidence of cancer in Nigeria had risen to 500,000 by the year 2015 (GLOBOCAN, 2016). Different observers had earlier predicted this figure to be reached by the year 2010 (Ogundipe and Obinna, 2008). Ferlay *et al.* (2012) also reported the pattern of cancer-related death in Nigeria; breast cancer 13,960 (19.5%), cervical cancer 8,240 (11.5%), liver cancer 11,663 (16.3%) and prostate cancer 9,628 (13.5%).

In the recent cancer statistics of GLOBOCAN for Nigeria, cancer accounts for over 78,000 deaths with an estimated 124,000 new cases annually (GLOBOCAN, 2020). These disparities or fluctuations could be a result of a lack of accurate cancer data, people not presenting to be screened, inadequate cancer registry or lack of access to

cancer care facilities among others. There are over 100 forms of cancer among which are cancer of the liver, pancreas, prostate, and breast.

2.1.2 Liver cancer

Liver malignancy is the 3rd foremost reason for cancer-associated death (GLOBOCAN, 2020). In 2018, it was predicted that liver cancer will be the 6th most reported cancer type and the 4th reason for cancer-linked death globally, accounting for an estimated 841,000 incidences and 782,000 mortalities yearly (Ferlay *et al.,* 2018). Ferlay *et al.* (2015) stated that liver malignancy is the second of the two most reported causes of mortality resulting from malignancy globally.

There was a 626,162 incidence of liver cancer in 2002, and 782,451 in the year 2012. Besides, this figure was said to increase by seventy per cent to 1,341,344 cases by the year 2035 (Ferlay *et al.,* 2015). The cancer statistics further revealed that the incidence and death rates in most regions of the world are two to three times more among men; however, liver cancer was listed as 5th and 2nd regarding global cases and mortality, respectively for males (Ferlay *et al.,* 2018). Furthermore, in transitional countries, the incidence rates are two-fold greater among men, while the greatest rates are seen predominantly in some lower human development index countries.

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma comprise between 75% to 85% and 10% to 15%, respectively of reported cases of primary forms of liver cancer (Bray *et al.,* 2018). However, the predominant predisposing factors for hepatocellular carcinoma are hepatitis viruses (B and C), high alcohol intake among others like tobacco use, aflatoxin-poisoned food, type 2 diabetes, and obesity (London *et al.,* 2018). These predisposing factors vary probably due to differences in region, race, age, etc. For example, China and eastern Africa are high-risk HCC regions with predominant predisposing factors being infection with HBV and aflatoxin. In Japan and Egypt, infection with HCV is reported to be a possible predisposing factor for liver cancer.

Meanwhile, in countries like Mongolia, a mix infection and other risk factors are seen as the reason for the high incidence of HCC (Chimed *et al.*, 2017). There was a report suggesting that the elevation in the burden of obesity has made some low-risk HCC

regions becomes high-risk HCC regions (Marengo *et al.,* 2016). However, while prevention has been a major way of curbing and reducing the incidence of HCC since 1982 globally through HBV vaccination that was first used in East Asia, there are no vaccination programmes against HCV (Chang *et al.,* 1997; Thursz and Fontanet, 2014; WHO, 2017; London *et al.,* 2018).

2.1.3 Pancreatic cancer

Pancreatic malignancy especially pancreatic ductal adenocarcinoma has been described as the most aggressive malignant tumour (Hafeez *et al.,* 2012) and ranks as part of the highly deadly neoplastic tumours with a five-year estimated survival rate of five per cent (WU *et al.,* 2014). Finding has it that, for pancreatic cancer, there is no known early detection method, and most cancer patients present with no visible symptoms. Hence, pancreatic cancer is not diagnosed until the cancer metastasises (Wolfgang *et al.,* 2013). Fewer than 20% of pancreatic cancer patients are entitled to surgery, unfortunately, most of them witness relapse, thereby necessitating alternative effective treatment and therapeutic options (Paulson *et al.*, 2013).

Furthermore, less than 7% of patients last pancreatic cancer within five years in the United Kingdom (Allemani *et al.,* 2018) and 8% in the United State (Siegel *et al.*, 2018). This is, however, the worst survival rate of all the twenty-two common cancers in the United Kingdom (Allemani *et al.,* 2018). Pancreatic cancer is the 5th biggest killer of cancer and the 3rd championing reason for cancer-linked mortality in the USA (Siegel *et al.*, 2018).

There were 9,921 and 8,912 newly diagnosed cases and death, respectively from pancreatic cancer in the UK in 2015 (ONS, 2015, 2017). However, nearly ten per cent of these patients are entitled to potentially curative surgery as a result of late presentation while those that are eligible for the procedure have around a 30% chance of living over 5 years (Neoptolemos *et al.,* 2017). Pancreatic cancer has been said to develop from pancreatic intraepithelial neoplasia that has the features of sequential aggregation of dysfunctions in the KRAS oncogene, and the downregulation of some tumour suppressor genes (Hruban *et al.,* 2000).

2.1.4 Some proteins implicated in cancer

2.1.4.1 Ki-67

Ki-67 nuclear protein (also called MKI67) has been described as a marker that is strongly linked to cell proliferation (Scholzen and Gerdes, 2000). This protein is seen in different stages of the cell cycle but for the quiescent state (Hohyun *et al.*, 2015). Reports have it that Ki-67 expression in cells indicates tumour multiplication rates and this corresponds to the initiation, progression, invasion, and poor tumour prognosis (Lee 1996).

There is reported agreement between the Ki-67 proliferation index and neuroendocrine tumours in relation to tumour size, biologic behaviour, and angioinvasion (Rindi *et al.*, 2006, 2007; Genc *et al.*, 2018a). There is, however, a recent study that describes the use of Ki-67 proliferation index to estimate the postoperative recurrence of pancreatic neuroendocrine tumours (Genc *et al.*, 2018b). Surprisingly, despite all the various reports on Ki-67 as a marker of cellular proliferation, scientists are still trying to unravel its physiologic function (Hohyun *et al.*, 2015).

2.1.4.2 p53

p53 is a transcriptional factor and is usually referred to as a tumour suppressor that operates in response to varying oncogenic stress (Figure 2.1). p53 induces cell-cycle abrogation and enhances regulated cell death to prevent cancer development (Vogelstein *et al.*, 2000). Usually, this transcriptional factor aggregates in the nucleus by a posttranslational mechanism which results in the activation of multiple downstream substrate genes including *p21* that carry out its tumour suppressor activities (Harris and Levine, 2005).

However, p53 can cause mortality through apoptosis to hinder a mutated DNA passage to the next progeny when there is a failure in DNA repair. Consequent to *p53* gene mutation, the cell supervision function of p53 protein could be compromised resulting in cell vulnerability and possible entry of the S phase with the damaged DNA. This might result in malignant cell change and tumour formation (Dong *et al.*, 2007). Nearly 37%-76% of the *p53* gene is usually altered in pancreatic cancer mainly via missense mutation (Scarpa *et al.*, 1993).

The alteration of this gene may be linked with the prevalence of this cancer type (Dong *et al.*, 2005, 2007), however, p53 is not the sole prognostic biomarker in surviving patients (Salek *et al.*, 2009).

2.1.4.3 $p21^{Cip1/Wap-1}$

p21 also called $p21^{Cip1/Wap-1}$ together with p27 and p57 are part of a family called Cip and Kip that inhibit cyclin-dependent kinase (CDK) (Abbas and Dutta, 2009). p21 protein has shown pleiotropic effects, especially on cell growth, survival, and apoptosis in both malignant and non-malignant cells (Gartel and Tyner, 2002). p21 protein performs several biological functions in the cell depending on the subcellular localisation and the differentiation stage (Liu *et al.*, 2013).

When present in the nucleus, p21 binds to and blocks the activity of several CDKs including the G1 - S phase, or G2 - to mitosis phase transition consequent to DNA injury, hence, p21 could act as a tumour inhibitor (Liu *et al.*, 2013). Also, p21 is very relevant in the initiation of replication senescence and stress-mediated premature senescence (SIPS) after injury to DNA (Cmielova and Rezacova, 2011). It was shown that p21 was elevated by hepatocytes growth factor stimulation via p53 protein induction (Inoue *et al.*, 2013).

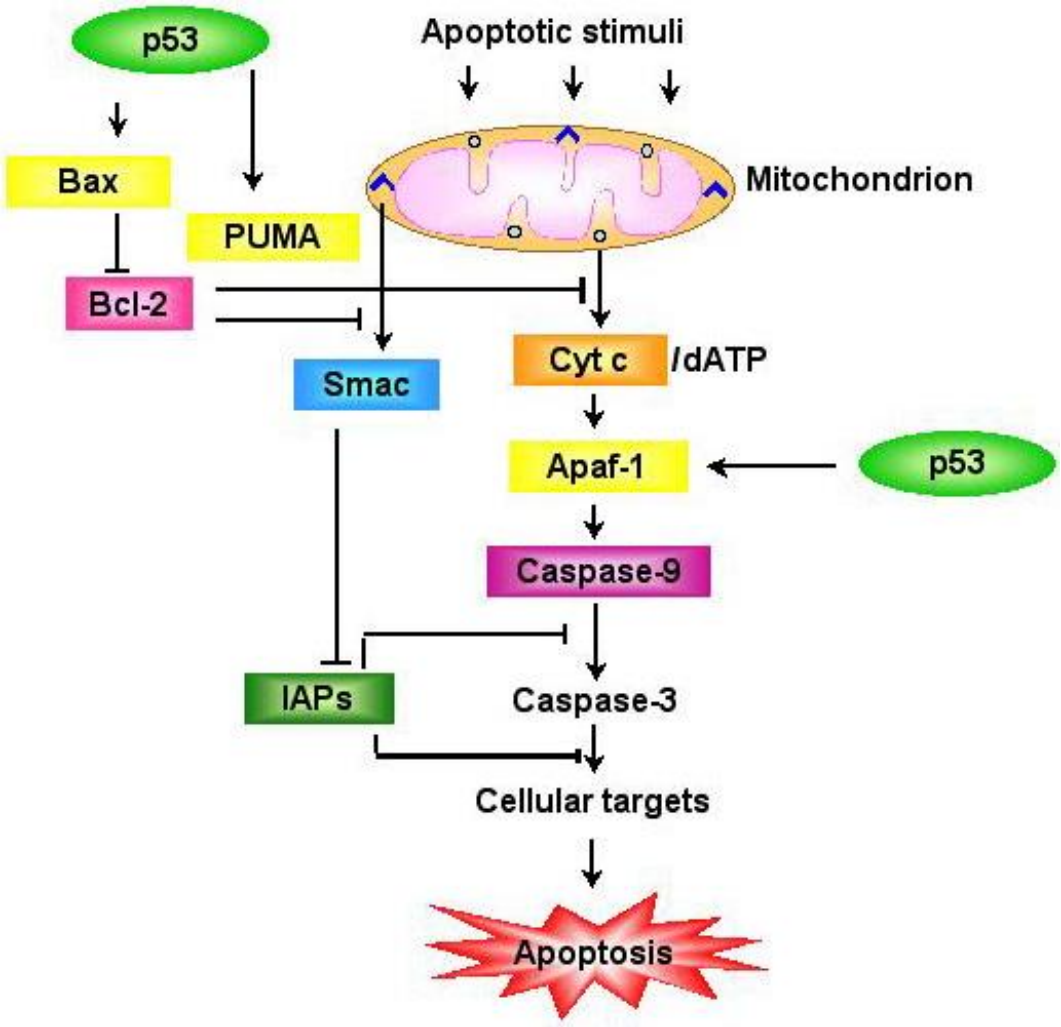


Figure 2.1. The involvement of p53 in apoptosis.

p53, a transcriptional factor, activates genes leading to apoptosis or cell cycle inhibition. However, when p53 binds through its binding sites in the regulatory section of p21, this inhibits the cycle through direct activation of the *Bax* gene. Bax protein overexpression induces apoptosis (Philomena, 2011).

2.1.4.4 Adenomatous Polyposis Coli (APC)

APC is a tumour silencer that regulates β-catenin, a major player in the Wingless-Int (Wnt) signalling (Sena *et al.,* 2006). APC is said to be a negative regulator of both beta-catenin and the Wnt signalling pathway (Korinek *et al.,* 1997). This activity takes place in the cytoplasm where APC together with other members of a complex inhibit the Wnt signalling and beta-catenin expression while also reducing its linkages with the hTcf-4 transcription factor (Korinek *et al.,* 1997).

APC behaves like a scaffolding molecule that enhances the assembly of the complex that contains casein kinase 1α, axin, and GSK-3β (Eisinger *et al.,* 2006). This complex enhances β-catenin phosphorylation, consequently its ubiquitin-mediated destruction. However, when there is a dysregulation of APC, beta-catenin aggregates in the cytosol, translocate into the nucleus, and link up with downstream transcription genes that enhance the function of Wnt signalling downstream substrate genes which induce cellular proliferation (Eisinger*et al.,* 2006).

2.1.4.5 Beta-catenin (β-catenin)

Beta-catenin has been described as a very important transcriptional factor with relevant roles in Wingless-Int (Wnt) signalling, cell development, stem cell renewal, and organ regeneration under normal physiological conditions (Lien and Fuchs, 2014). Dysregulation of β-catenin and its abnormal functions is found in many cancer cells and is responsible for the malignant transformation of normal cells (Morin, 1999).

In a normal physiological state, the cytoplasmic accumulation of beta-catenin is kept low and controlled via a complex of protein inhibitors that comprises APC/GSK-3β/axin. This complex performs a continuous phosphorylation-induced ubiquitin-mediated degradation of β-catenin thereby preventing its nuclei movement (Dajani *et al.,* 2003). However, Wnt signalling activation will result in GSK-3β phosphorylation, consequently APC/GSK-3β/axin complex dissociation. This will prevent β-catenin degradation and allows for its cytoplasmic accumulation, and eventually, its nuclei translocation.

This process allows β-catenin to interact and activates several downstream transcription factors including TCF/LEF. β-catenin controls various effector genes

transcription that is linked to cellular survival, growth, proliferation, and invasion (Dajani *et al.*, 2003; Jeong *et al.*, 2012).

2.1.4.6 Caspase-3

The caspases belong to the cysteine protease class that performs vital functions in cell death through their interactions with key cellular proteins (Devarajan *et al.*, 2002). Both caspase-3 (otherwise called apopain) and caspase-8 are examples of the 14 identified caspases in mammals (Johnstone *et al.*, 2002). A report has it that caspase-8 is a major player in death receptor-induced apoptosis cascade together with the TNFR and Fas/FasL that could promote cleavage and activation of several target-caspases like caspase-3 (Ashkenazi and Dixit, 1999).

In addition, caspase-3, aside from being activated by the death receptor or caspase-9/mitochondria-mediated pathway, was suggested to take part in the late destructive stage of cell death (Vaughan *et al.*, 2002). Although procaspase-3 is localised in the cytosol, caspase-3 has played important roles at various stages of apoptosis, especially through its nuclear substrate that is involved in nuclear morphological changes (Green, 1998; Vaughan *et al.*, 2002).

Kerr *et al.* (1972) previously said that the whole process of cell death is genetically regulated. However, dysregulation of this process is linked to several conditions (Soengas *et al.*, 2001). Caspase's activities could be stimulated either by extracellular or intracellular factors (Nicholson, 1999). Caspases can then be allotted into two broad classifications depending on their role in apoptosis. A caspase could therefore be an initiator caspase (caspase 8, 9, 10), or an executioner caspase (caspase 3, 6, 7) (Nicholson, 1999). When the initiator caspases are activated by apoptotic signals, they consequently activate downstream executioner caspases.

However, executioner caspases catalyse specific cleavage of several important cellular proteins after their activation, (Cryns and Yuan, 1998; Thorneberry and Lazebnik, 1998) and eventual events like membrane blebbing, condensation of chromatin, and DNA fragmentation. Enari *et al.* (1996) reported that caspase-3 participates effectively in the completion of apoptosis with a supporting view from other research work carried out in mice (Kuida *et al.*, 1996).

2.1.4.7 Alkaline phosphatase

Alkaline phosphatase (ALP) is a crucial index of differentiation in neoplastic tumours (Dabare *et al.*, 1999). Differentiating agents were found to upregulate ALP activity in malignant cells (Gianni *et al.* 1993; Lenhard *et al.* 2000). For example, all-*trans*-retinoic acid (ATRA) performed this role through ATRA receptors RAR/RXR (Lenhard *et al.* 2000). However, stimulation of differentiation in pancreatic cancer by a specific inhibitor of DNA methylation (MIA PaCa-2 cells) promotes ALP expression (Yamada *et al.*, 1996). Also, ATRA was said to upregulate alkaline phosphatase activity in Panc-1 cells (Guo *et al.*, 2006).

2.1.4.8 B-cell lymphoma 2 (Bcl-2)

Bcl-2 is regarded as a core actor in the mitochondrial cascade of controlled cellular death. They localise directly to this organelle and take part in the regulation of membrane permeabilisation (MOMP) (Popgeorgiev *et al.*, 2011). Bcl-2-associated proteins equally can localise in other subcellular components like the nucleus, Golgi apparatus, endoplasmic reticulum, lysosome, and peroxisomes (Guan *et al.*, 2015) (Figure 2.2).

The proteins regulate MOMP and also carry out crucial cellular activities like cell cycle control, migration, and calcium homeostasis (Popgeorgiev *et al.*, 2011). An earlier report has it that Bcl-2 performs a paradoxical role in the cells and this ability to interchange roles depends on the protein localisation (Portier and Taglialatela, 2006). Meanwhile, Bcl-2 are globular proteins containing alpha-helices with unique conserved B-cell lymphoma 2 homology (BH 1 - 4) domains. There are three B-cell lymphoma 2 family subgroups: apoptosis inhibitors [Bcl-2, BclxL, Mcl-1 (with all 4 BH domains)], apoptosis promoters [Bax, Bak, Bok (with 3 BH domains), and the apoptosis promoter BH3- alone subgroup [Bad, Bid, Bim, Bik (with a single BH3 domain)].

Furthermore, several B-cell lymphoma 2 proteins have a water-repelling-transmembrane anchoring domain which makes the proteins adhere to intracellular membranes. Bax and Bak proteins can form oligomers and also activate MOMP; this is an irreversible phase in apoptosis. Bcl-2 binds directly to Bax and Bak to inhibit the

MOMP. Proteins with BH3 alone regulate this activity by activating Bax and Bak or suppressing anti-apoptotic B-cell lymphoma 2 proteins (Youle and Strasser, 2008).

There were also reports showing the ability of B-cell lymphoma 2 to regulate the works of mitochondrial inner membrane proteins like cyclophilin D via direct linkages. This paradox was clarified; which suggests the possibility of Bcl-2 being found at the midway of the inner and outer membranes (Nguyen *et al.,* 1993).

Some homologs are resident in the ER and nucleus (Choi *et al.,* 2016). However, B-cell lymphoma 2 was noticed to translocate to the nucleus also, and this process relies on the Thr56 phosphorylation state. B-cell lymphoma 2 participates in multiprotein complex activities in the cell nucleus (Barboule *et al.,* 2005, 2009) and a report suggested that its nuclear localisation was pro-apoptotic (Bryce and Giulio, 2006).

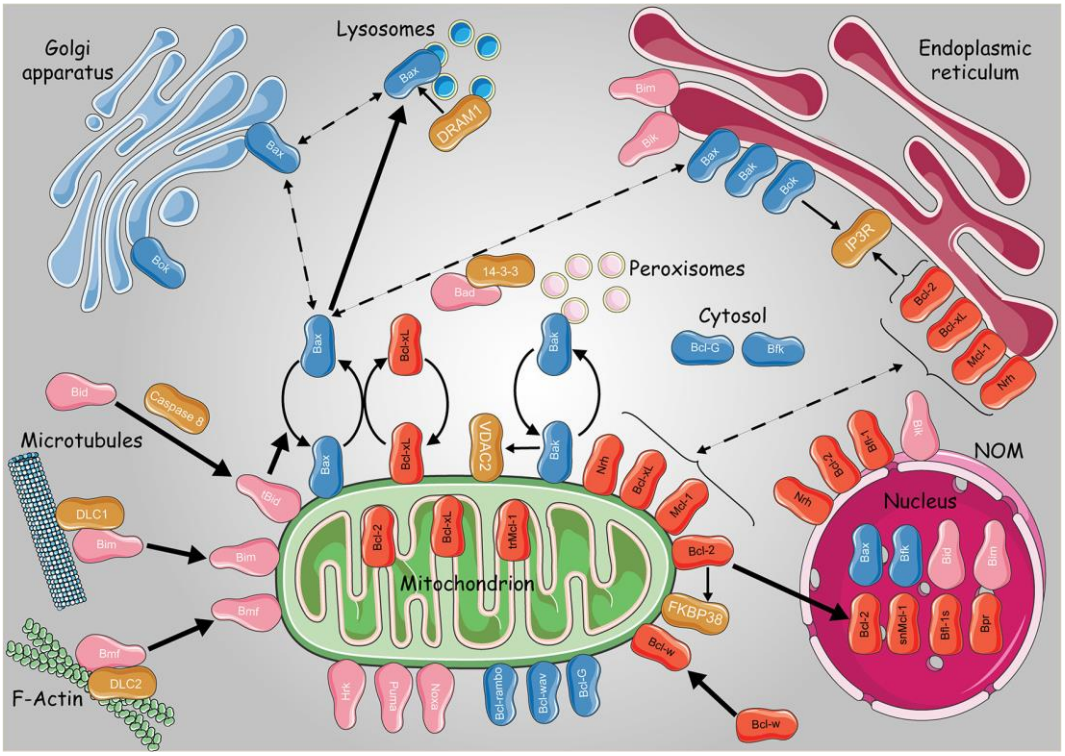


Figure 2.2. B-cell lymphoma 2 subcellular dynamics.

The domains of apoptosis inhibitors (red), apoptosis promoters (blue), and apoptosis promoter BH3-only (pink) proteins are represented including the not-Bcl-2 homologs (deep orange boxes). Bold lines (translocation of protein in stress states), continuous lines (translocation of protein in normal states). Some features therein were obtained from www.servier.com (Popgeorgiev *et al.*, 2011).

2.1.5 Antioxidant

Body cells possess effective and efficient endogenous antioxidant apparatus that hinder or permit recovery from oxidative injury or stress. However, there are small molecular weight antioxidants like vitamins, catalase, and glutathione (Rautalahti and Huttunen, 1994; Seung *et al.,* 2007). These enzymes help to defend the body against oxidative stress (Chen, 2012). Goodarzi *et al.* (2018) reported that these antioxidants could be classified as either externally (derived from diets containing antioxidant substances e.g. vitamins, minerals, polyphenols) or internally (derived from the actions of body enzymes like catalase) generated.

Meanwhile, for cellular health, there must be adequate equilibrium between reactive oxygen species (ROS) recruitment and antioxidant guard apparatus, while derangement in this balance could induce oxidative injury or stress. Vitamins (C, E) and selenium have been utilised as antioxidants against oxidative stress induced by toxicants in experimental models (Bahmani *et al.*, 2016; El-Shafei and Saleh, 2016).

β-carotene and polyphenolic compounds, such as resveratrol were potent as antioxidants in disease states (Revin *et al.,* 2019). However, intracellular ROS oxidises lipids, proteins, and nucleic acids such as DNA, which results in damage or injury to various cellular organelles (Zhang *et al.,* 2016). There is also a higher level of ROS in the microenvironment containing limited nutrients for malignant cells relative to normal cells (McCarthy, 2011).

Meanwhile, there is high generation of ROS in pancreatic cancer cells (Li *et al.,* 2011a), while ROS promotes DNA damage, facilitates cell survival, enhances the initiation of cancer formation and progression, and the transformation of the malignant cells (Zhang *et al.,* 2016). Excessive ROS promotes the cytoplasmic presence of cytochrome C thus triggering cell death (Fruehauf and Meyskens Jr., 2007). However, redox homeostasis regulation is vital to cell function maintenance, and survival. Malignancies are said to be evidenced by an elevated amount of ROS and the strategies to decrease them were said to be an effective way in cancer therapy (Zhang *et al.*, 2016).

Fortunately, various strategies have been documented in the control of cancer by targeting these oxygen metabolites (Zhang *et al.*, 2016). Studies on some types of malignancies such as breast cancer have shown that enhancement of antioxidant enzyme function can inhibit cancer cell growth (Church *et al.*, 1993).

Meanwhile, GST-pi expression was reported in physiological human epithelial tissues (Sarkar *et al.*, 1977; Terrier *et al.*, 1990). There were suggestions that an increase in GST-pi activity correlates with malignant transformation, resistance to anticancer agents, and poor prognosis (Ribrag *et al.*, 2003) while its downregulation or inhibition in T cell line and rat hepatoma cells favours apoptosis (Asakura *et al.*, 2001). However, catalase has been regarded as one of the two primary cellular antioxidant enzymes, aside from superoxide dismutase (Li *et al.*, 2011a). Catalase participates in cell defence to hinder oxygen metabolites (Glorieux *et al.*, 2015) through the dismutation of H_2O_2 into H_2O and O_2.

There are reports that stated that catalase also performs some decomposition (Kono *et al.*, 1998; Heinzelmann and Bauer, 2010), oxidation (Brunelli *et al.* 2001), marginal peroxidase (Johansson and Borg, 1988), and low oxidase activities (Glorieux *et al.*, 2015; Doskey *et al.*, 2016). Meanwhile, a reduced amount of catalase activities in malignant cells was reported (Lewis *et al.*, 2005). Several therapeutic compounds have been documented to increase catalase activity (Suzuki *et al.*, 2000; Klingelhoeffer *et al.*, 2012).

2.2 Arsenic

Arsenic, a well-known heavy metal with medicinal uses for decades has received much attention due to the dysfunctions it causes in man and animals including plants (Bhadauria, 2019). Man and animals come in contact with arsenic mainly via oral or nasal routes (Bates *et al.*, 1992). Oral contact mainly takes place via the consumption of arsenic-exposed drinking water, food, and drugs. Nasal contact also takes place through breathing in arsenic-polluted air e.g. from semiconductor and glass manufacturing industries, burning of coal, non-ferrous ore smelting (Liu *et al.*, 2001).

The assessment of challenges arising from exposure to this pollutant in drinking water is a pertinent topic confronting the scientific world. Some countries have higher

amounts of arsenic in their consumable water (Chatterjee *et al.*, 1995; NRC, 1999). It has become clear that the reduced amount of arsenic in contaminated water typically seen in a few countries may present a huge health risk to man (Chatterjee *et al.*, 1995; NRC, 1999).

Several decades ago, arsenic was reportedly administered per os as Fowler's solution in tonic preparations and as a therapeutic agent against blood and respiratory disease (Leslie and Smith, 1978). In addition, arsenic has been utilised against syphilis (Moore, 1933), Lichen planus, Verruca planum, and psoriasis (Goodman and Gilman, 1942) when given parenterally. Chisholm, (1970) has also reported the domestic, agricultural, and industrial benefits of arsenic as an insecticide, weedicide, rodenticide, and arsine. However, the frequent utilisation of this metalloid in these forms are typically challenging the animal and human environment

However, arsenicals were reported to cause chronic hepatitis and hepatic cirrhosis when consumed in arsenic-contaminated beer (Wolf, 1974) and enhance liver enzyme activities in cattle (Rena *et al.*, 2010). Non-skin attributes of chronic arsenic contamination via the drinking of arsenic-polluted water were documented (Tseng, 1977). Chronic arsenic dermatosis that resulted from the consumption of arsenic-poisoned well water was first reported by Saha (1984).

FAO and WHO (1983) jointly reported that arsenic in its inorganic forms possesses a higher toxicity level compared to the less toxic organic forms. Examples of some well-tolerated organic arsenic in living organisms are arsenobetaine (AsBet) and arsenocholine (AsChol), (FAO and WHO, 1989). Moreover, arsenic such as As^{3+} or As^{5+} is ubiquitously and naturally available in the environment. Arsenic poisoning in experimental animal model contributes to hepatic tumour development (Waalkes *et al.*, 2000) and other reproduction-related activities (Chattopadhyay *et al.*, 1999, Samuel *et al.*, 2017), and increased levels of corticosterone and adrenocortical steroidogenesis (Ghosh *et al.*, 1999), and severe metabolic derangements in human (Tseng *et al.*, 2002).

Acute arsenic poisoning may result in gastric and intestinal tract abnormalities (Goebl *et al.*, 1990), while chronic contact may cause higher degrees of changes to the

cells like degeneration, inflammation, and neoplasia (Naiger and Osweiler, 1989). Generally, arsenicals are contaminants with deleterious effects in the kidney, uterus, liver, lungs, and bladder (NRC, 1999; Waalkes *et al.*, 2000, 2003).

Meanwhile, sodium arsenite a derivative of arsenic is abundant in the ecosystem through different human activities (Chatterjee *et al.*, 1995). Among the several inorganic arsenical compounds, arsenite (As^{3+}) and arsenate (As^{5+}) combined well with O_2 and Na (Hughes, 2002). $NaAsO_2$ is considered the most toxic because of its affinity for endogenous thiol components and suppression of crucial biochemical pathways (Hughes *et al.*, 2011). Similarly, humans and animals are exposed to sodium arsenite via various channels (Waalkes *et al.*, 2003).

Arsenic poisoning was reportedly linked to sperm toxicity (Pant *et al.*, 2004; Samuel *et al.*, 2017), and morphological derangement of the male reproductive tissues (Sarkar *et al.*, 2003; Samuel *et al.*, 2017). However, the pathway of male reproductive organ toxicity from arsenic exposure remains unclear. Some pollutants exert their anti-gonadal activities by direct abrogation action on the male sex organ and the pituitary gland leading to changes in gonadotrophin concentrations and consequently impaired spermatogenesis (Sarkar *et al.*, 2003).

2.2.1 Clastogenicity

Clastogenicity is the process whereby there is a structural change such as breakage or damage to the chromosome. Everson *et al.* (1988) and Maurici *et al.* (2005) emphasised that these changes could result in deletion, addition, or rearrangement of the gene sequence of the chromosome as the process of exchange in which homologous chromatid strand interchange and reunite during DNA replication. However, substances or compounds of plant, physical or chemical origin could cause these changes.

2.2.2 Genotoxicity

Genotoxicity refers to alterations in the architecture or number of genes via the interaction of chemicals or substances with the genetic material and/or non-genetic material targets like the spindle apparatus (Maurici *et al.*, 2005). Genotoxicity is an inherent attribute of some agents that allows them to be inimical to the genetic

materials in organisms (Savale, 2018). With different factors affecting DNA, RNA, and other genetic materials, the attribute of genotoxicity only applies to agents that produce alterations in genetic information (Savale, 2018). Any agent with the features of genotoxicity is referred to as a *genotoxin.* Genotoxins can have three primary effects on organisms when genetic information is affected (Maurici *et al.,* 2005). Genotoxin can be a carcinogen, mutagen, or teratogen (Savale, 2018).

Some genotoxins are carcinogenic because they affect cancer-inhibiting genes, thereby leading to cancer development (Maurici *et al.,* 2005). Genotoxic agents can also cause mutations in body cells leading to uncensored division and growth uncontrollably. Genotoxin can equally have damaging effects on different proteins and suppress other agents that usually hinder these processes. The inability of those agents to hinder these processes in some cells could result in mutation, and uncontrolled cell division and growth (Maurici *et al.,* 2005).

More often than not, genotoxicity will lead to mutations in many cells and mutation causing a lot of genetic diseases including cancer. Although, on very few occasions gene mutations are harmless and can proceed unnoticed while some mutations can lead to birth defects (Maurici *et al.,* 2005). Mutagenicity is considered to be the stimulation of transferrable alterations in the architecture of cell genetic material (Maurici *et al.,* 2005; Savale, 2018). Mutagenicity/genotoxicity test is an *in vitro* test system employed severally in carcinogenicity prediction.

2.2.3 Carcinogenicity

Carcinogenesis is recognised as a process with several steps such as cancer initiation, promotion, and malignant progression (Weston and Harris, 2013; Basu, 2018). A carcinogen is a chemical agent or a mixture of such compounds that elicit malignancy or enhance cancer development (UNECE, 2004). Alternatively, carcinogens are substances that cause tumours (benign or malignant), enhance the occurrence of malignant transformation, or reduce tumour occurrence time when exposed (Maurici *et al.,* 2005). These agents could be genotoxic or non-genotoxic depending on the pathway.

Genotoxic carcinogens start malignancy by interacting with DNA. This interaction can lead to DNA damage or chromosomal aberrations (Butterworth, 1990; OECD, 2006). Non-genotoxic carcinogens can cause indirect alterations to the architecture, number, or function of DNA that may produce changes in gene function (Butterworth, 1990; OECD, 2006). However, there are other reported classifications of carcinogens (Latarjet, 1960; Currie and Currie, 1982; Darnell *et al*; 1990).

Physical Carcinogens: Darnell *et al.* (1990) reported that this form of carcinogens is mainly due to ionising radiations such as x-rays or gamma radiations and atomic particles. However, this form of carcinogen is linked with some cancer types of the skin, lungs, ovary, and blood in both humans and animals.

Biological Carcinogens: Latarjet, (1960) described some organisms such as viruses as having a link to cancer. These viruses are of two types: the DNA tumour viruses (hepatitis B or papillomavirus) and the RNA tumour viruses (retroviruses) like the hepatitis C virus. However, other organisms like parasites are also grouped as biological carcinogens, for example, *Schistosoma haematobium*, and *Clonorchis sinensis*.

Meanwhile, Currie and Currie, (1982) described the third group as chemical carcinogens. This is further sub-classified into direct-acting carcinogens: these chemical carcinogens (highly reactive metabolites which do not require metabolic activation to cause cancer but rather react directly with cells and induce cancer e.g. nitrogen benzyl chloride, indirect-acting (pro-carcinogen) (require metabolic activation to become active e.g. polyaromatic hydrocarbons and co-carcinogens; promoting agents (possess little or no properties of direct-acting carcinogens or pro-carcinogens. Several mutagens are considered carcinogenic (Maurici *et al.*, 2005), and tumour-causing agents in animals are reportedly hypothesised to be human carcinogens (UNECE, 2004).

2.2.4 Mechanism of arsenic-mediated carcinogenesis

The metabolism and biotransformation of arsenic are suggested to be responsible for its cancer-causing ability (Ebert *et al.*, 2011) (Figure 2.3). However, inorganic arsenical compounds are easily tolerated by the gastrointestinal tract when consumed

via drinking water (Pomroy *et al.,* 1980). Upon ingestion, the compound mainly in its As^{5+} form navigates the cells via membrane-associated proteins such as transporters or channel proteins (Wang *et al.,* 2004, 2007; Dilda and Hogg, 2007). This arsenate through a GSH-dependent reduction reaction is catalysed (polynucleotide phosphorylase, mitochondrial adenosine triphosphate synthase) into a more toxic As^{3+} form in the cell (Nemeti *et al.,* 2010). Both arsenite and its methylated conjugates as glutathione conjugates are translocated from the primary liver cells into bile (Kala *et al.,* 2000).

Mono - and dimethylated As^{3+}species migrating from the hepatocytes are reactive with the ability to cause injury in various organs. Furthermore, the injury takes place majorly via the generation of ROS together with GSH depletion (Thomas *et al.,* 2001). This adverse effect of As^{3+} is linked to its high covalent interaction with the thiol groups, and this interaction with protein often results in protein inactivation or degradation (Dilda and Hogg, 2007).

2.2.5 Arsenic as a co-mutagen

Arsenic in its inorganic forms does not have direct interaction with genetic materials, hence is not clastogenic or mutagenic at low dosages (Klien *et al.,* 2007). However, this does not apply to the metabolites and other components derived from the metabolic process (Rossman *et al.,* 2011). Meanwhile, reduced dosages of this metalloid can potentiate mutagenesis via some cancer-causing agents such as diepoxybutane, N-Methyl-N-nitrosourea, UV light, and sulfonate (Rossman *et al.,* 2004).

2.2.6 Arsenic-induced DNA damage

Arsenic-mediated ROS production is derived by either cycling of As^{3+} and As^{5+} (Flora, 2011) or via alteration of the mitochondrial electron transport chain (Rossman, 2003) (Figure 2.3). The latter mechanism is responsible for the majority of the arsenic-linked mechanisms of ROS recruitment. Mitochondrial reactive oxygen species are derived via monomethylarsonous acid (MMAIII)-directed suppression of mitochondrial complexes two and four (Barrett *et al.,* 1989). This yields a backlog of electrons, consequently leading to the leakage of electrons via complexes one and three (Naranmandura *et al.,* 2011).

The release of these electrons consequently results in superoxide anion (O_2•−), H_2O_2, and hydroxyl (OH•) radicals' accumulation (Wang *et al.*, 2004, 2007). Arsenic-directed generation of free radical species is linked to some biochemical events (Martinez *et al.*, 2011) (Figure 2.3). This metalloid could induce reactive N_2 species (RNS) formation such as peroxynitrite but the pathways involved are not understood (Gurr *et al.*, 2003). Meanwhile, nitrogen metabolites elevation resulted in oxidative DNA damage, DNA alkylation, and deamination (Wink *et al.*, 1991).

2.2.7 DNA repair processes and arsenic

Arsenic affects the genetic material repair capacity of cells. In Figure 2.3, this can be via alteration in nucleotide- (NER) and base-excision repair (BER) cascades. This metalloid affects the NER repair mechanism by lowering the number of the incision steps (Hartwig *et al.*, 1997), downregulates NER-associated genes level, and the amount of XPC protein (Nollen *et al.*, 2009).

Furthermore, methylated arsenite species obtained from the metabolism process hinder PARP1 (NER promoter) activity (Walter *et al.*, 2007). Arsenic metabolites also reduce the levels of BER-associated genes like DNA ligase IIIα (Ebert *et al.*, 2011). This was also seen in murine lung tissue treated with arsenic (Osmond *et al.*, 2010).

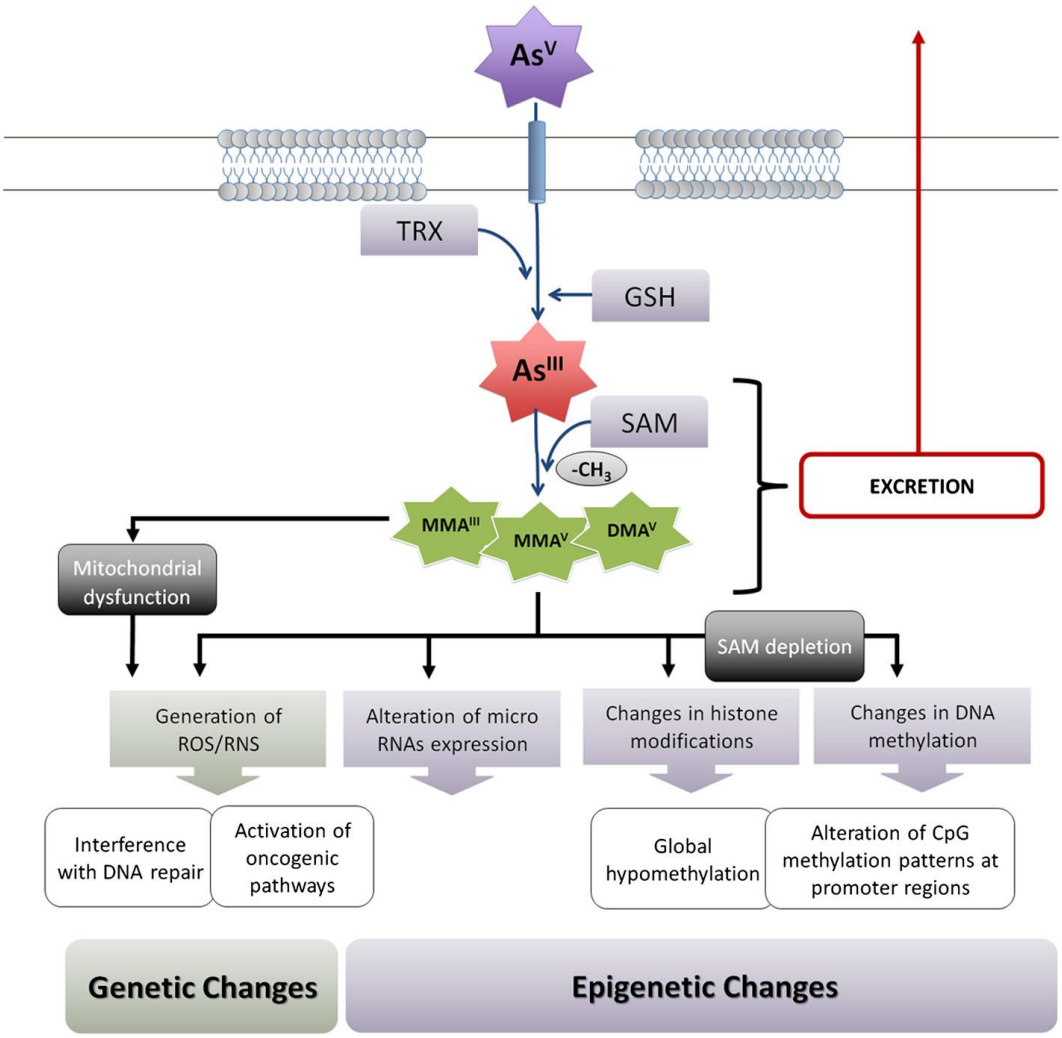


Figure 2.3. Mechanistic information on arsenic-mediated carcinogenesis.

The arsenic biotransformation process is responsible for its carcinogenic potential via genetic and epigenetic changes. This process occurs via a series of cycles of oxido-reduction, and methyl group donation. Arsenateis reduced to arsenite by utilising GSH and TRX as electron donors. Arsenite is methylated using the methyl groups sourced from S-Adenosyl methionine (SAM). This methylated arsenite or metabolite has a strong carcinogenic ability (Hubaux *et al.*, 2013).

2.2.8 Arsenic, chromosomal, and genomic instability

Micronuclei formation alongside chromosomal aneuploidy was significantly elevated in arsenic-treated cells. This resulted from the impact of the metalloid on the sulfhydryl component of some proteins such as tubulin (Zhao *et al.,* 2012). However, p53-dependent elevation in the p21 level was reported in normal cells after DNA injury was impaired in arsenic-exposed cells. This consequently resulted in cell cycle progression (Komissarova and Rossman, 2010).

Correspondingly, arsenic-mediated alteration of PARP1 activity plays a part in genomic instability (Walter *et al.,* 2007). Martinez *et al.* (2010) compared DNA disruptions in arsenic-treated and untreated lung tumour cells. They indicated that the site and frequency of these disruptions differ in both cases vis-a-vis genomes of lung tumours from subjects that did not smoke and those persistently exposed to the metalloid have segmental amplifications and losses of DNA in a chromosomal locus. Meanwhile, 19q13.33 genes are reportedly oncogenic in an animal model (Parsons *et al.,* 2007).

2.2.9 Arsenic, histone modification, and gene expression

Jo *et al.* (2009) reported that arsenic mediates the lowering of H4K16 acetylation levels. A modified H3K (4, 9, and 27) histone methylation was noticed in cancerous and non-cancerous lung cells treated with arsenic. This resulted in the lowering of epigenetic modification-associated gene levels (Zhou *et al.,* 2008). This metalloid was said to change the chromatin scenery of arsenic-mediated malignant cells via the mechanism that results in the upregulation of Wnt signalling (Jensen *et al.,* 2009).

2.2.10 Arsenic, epithelial-to-mesenchymal transition, and micro-RNA

Epithelial cells from human bronchial were used to demonstrate that p53-knock-down cells treated with chronic arsenic-mediated malignant transformation coupled with epithelial-to-mesenchymal shift (Wang *et al.,* 2011). A reduced activity level of a miR-200 gene was related to this exposure and proven to take place via elevated promoter methylation. However, the restoration of the miR-200b levels completely reversed and hindered the arsenic-mediated EMT and malignancy (Wang *et al.,* 2011). Arsenic exposure can change the amount of *in vitro* and *in vivo* miRNA, and downregulates miR-9, -181b, -124, and -125b expression levels in the chick embryo. Reduction in

miR-9 and miR-181b level led to increased activity of Nrp1 that activated angiogenesis, cell migration, and tumour formation (Cui *et al.,* 2012). Arsenite mediated the upregulation of various miRNAs like miR-222, and this upregulation was reversed when normal folate levels were reestablishment (Marsit *et al.,* 2006).

2.2.11 Arsenic and epigenetic alterations

Arsenic metabolism depletes S-Adenosyl Methionine (SAM) level thereby causing aberration in the methylation of DNA. The methyl groups obtained from SAM have been used to detoxify arsenic (Figure 2.3), and the reduced level of these methyl groups will produce arsenic-associated epigenetic changes (Simeonova and Luster, 2000). The level of SAM in the cells is usually not altered, but the increased need for SAM resulting from chronic arsenic contact will affect the number of methyl groups available in the cell (Mazumder, 2005).

S-Adenosyl methionine is the major contributor of the CH_3 group to DNA-methyltransferases. However, the reduction in methyl groups level in the cell can result in hypomethylation and alteration in chromatin remodeling (Intarasunanont *et al.,* 2012). These epigenetic modifications enhance malignant transformation in different types of cells (Reichard and Puga, 2010).

Hypomethylation was noticed in lymphoblastoid cells exposed to arsenic (Intarasunanont *et al.,* 2012). Meanwhile, low doses of arsenic have produced DNA hypomethylation in an animal model (Zhao *et al.,* 1997). Also, arsenic-mediated SAM reduction can change the status of CpG methylation of promoters or promoter regions of target genes like *p*53, and promoter hypermethylation of tumour suppressors (Chanda *et al.,* 2006). Oxygen metabolites obtained during the arsenic biotransformation process played a role in methylation (Ziech *et al.,* 2011).

2.2.12 Arsenic and EGFR

EGFR (epidermal growth factor receptor) locus is a possible location where a mutation can occur which consequently will lead to impairment in the EGFR pathway. This impairment could cause structural changes that may produce imbalances in the autoinhibitory loop of the EGFR. The receptor can be forced into a constitutive active site that is not ligand-dependent (Yarden and Sliwkowski, 2001). This EGFR

constitutive active state can be mediated by arsenic as reported (Sung *et al.,* 2012) (Figure 2.4).

Arsenic activation of c-Src can produce ligand-independent EGFR phosphorylation and constitutive stimulation. c-Src physically interacts with EGFR thereby leading to two special tyrosine phosphorylation cascades (Tyr845, Tyr1101) (Simeonova and Luster, 2002). This metalloid is also involved in the activation of components of the EGFR cascade such as Ras via reactive oxygen species (Li *et al.,* 2011b). Cheng *et al.* (2004) reported the inhibition of STAT3 by arsenite via JAK suppression.

However, it was documented that arsenite upregulates STAT3 via c-Jun NH2 kinase (JNK) thereby activating Akt (Liu *et al.,* 2012). The activity of EGFR and some proteins such as Rac1 GTPases have been reportedly activated in cell lines by arsenic (Herbert and Snow, 2012; Sung *et al.,* 2012). However, targeting this pathway in the bid to impair the pathogenesis of diseases is already in place (Cheng *et al.,* 2012).

2.2.13 Arsenic and PI3K/AKT signalling

PI3K/AKT signalling begins with receptor tyrosine kinases (RTK's) activation via interaction with extracellular growth factor consequently the activation of downstream substrates which are involved in growth, survival, and cell multiplication (Papadimitrakopoulou, 2012). Arsenite possesses the ability to phosphorylate the PI3K/AKT cascade (Chen *et al.,* 2012) (Figure 2.5).

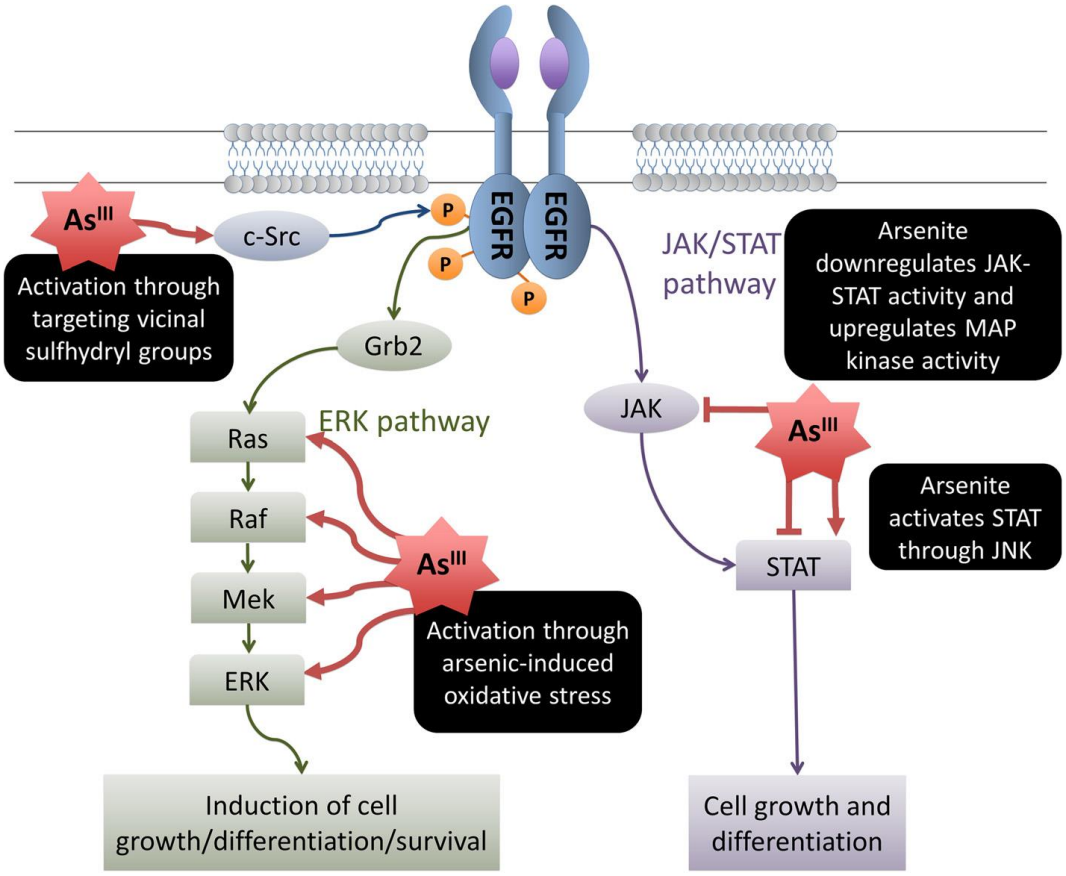


Figure 2.4. Arsenic stimulates EGFR activity.

Arsenic stimulates the activity of EGFR and part of its components in cells. Arsenic consequently activates AKT via STAT3 induction through JNK (Hubaux *et al.*, 2013).

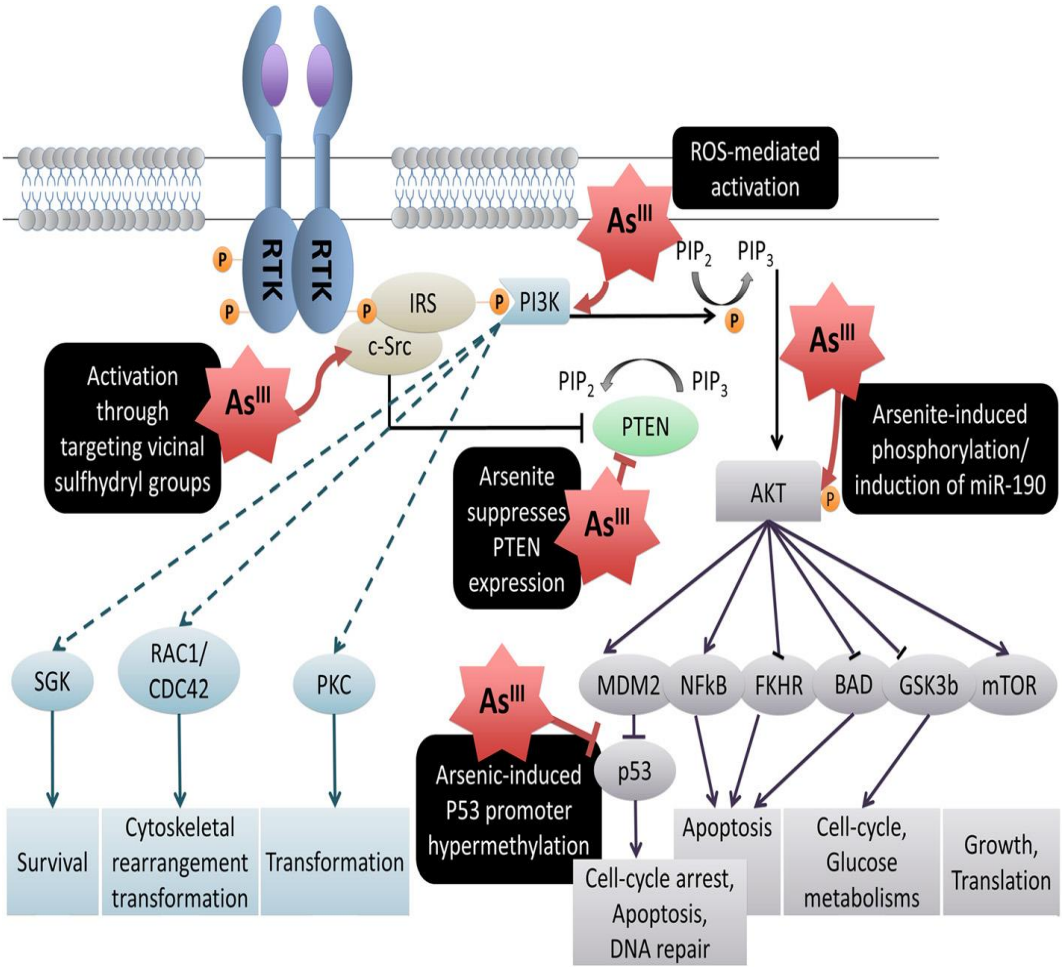


Figure 2.5. Arsenic stimulates PI3K/AKT signalling.

Arsenic participates in PI3K/AKT signalling activation via receptor tyrosine kinase and the target proteins PI3K (light blue), AKT (grey), and the consequent events are shown (Hubaux *et al.*, 2013).

2.2.14 Arsenic and Nrf2-KEAP1 signalling

Nrf2 has a leucine-zipper DNA binding site that possesses the ability to interact with antioxidant and electrophile response elements. In a normal physiological state, the transcription factor is usually controlled by KEAP1 and positioned for proteolytic degradation (Zhang, 2006). However, due to oxidative stress or other forms of stress, Nrf2 will usually respond by separating from Enhanced at Puberty 1 (EAP1), and translocating to the nucleus. Meanwhile, an aberration in the KEAP1 E3-ubiquitin ligase complex could result in NF-κB stimulation (Thu *et al.,* 2011).

Nrf2 pathway stimulation was proposed to abrogate both arsenite and MMA III-mediated toxicities and pathology (Wang *et al.,* 2008). Zhang (2006) suggested the possible participation of the metalloid in Nrf2 stabilisation via its affiliation with KEAP1 that enhances the disruption of the Nrf2-KEAP1-CUL3 complex (Figure 2.6) (Andujar *et al.,* 2010).

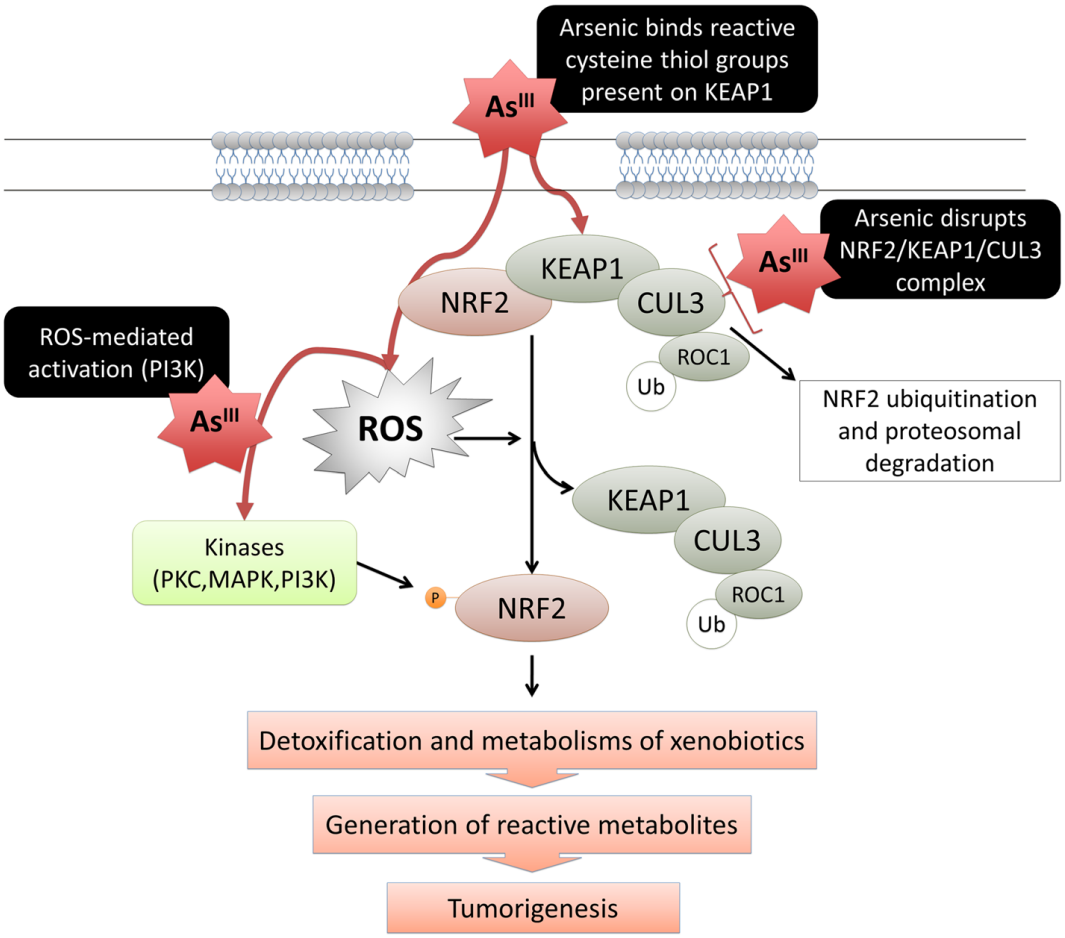


Figure 2.6. Arsenic influences Nrf2 signalling.

Nrf2 regulates oxidative stress response activation. The stimulation of the Nrf2 pathway was proposed to abrogate both arsenite and MMA III-mediated toxicities and pathology (Hubaux *et al.*, 2013).

2.3 *Tridax procumbens*

2.3.1 Classification

Table 2.1. Taxonomy of *Tridax procumbens*

Plantae (Plant)/Tracheobionta	Kingdom/Subkingdom
Magnoliophyta	Division
Magnoliopsida/Asteridae	Class/Subclass
Asterales/Asteraceae	Order/Family
Tridax	Genus
Procumbens	Species
Tridax procumbens Linn.	Binomial name

(Mir *et al.*, 2017)

2.3.2 Names and languages of *Tridax procumbens*

Tridax procumbens has been called various names in different languages and countries of the world. This could be attributed to the various functions it serves. For example, the plant is called Ewe Ehoro among the Yoruba people of western Nigeria because it is used as rabbit feed (Olowokudejo, 1987).

2.3.3 Morphological description of *Tridax procumbens*

Tridax procumbens is a commonly found procumbent weed that can be grown in an open space and could thrive in many places such as in coarse-textured soil (Khan *et al.,* 2008). It has a stunted yellow colour corolla from a hairy blade-like leaf. The leaves of *Tridax procumbens* are simple, opposite, exstipulate, lanceolate, and ovate. The leaves are base-wedge shaped, 3-7cm long, with irregularly toothed margins and hairy on both surfaces.

Tridax procumbens stem is usually ascending in height (up to 0.3 - 0.5m) with diverged sparsely hairy and rooting at its nodes. *Tridax procumbens* flowers are yellow, tubular, and hairy with an inflorescence capitulum. There are two major forms of its flower (ray and disc florets. They have basal placentation that undergoes fruiting throughout the year (Khan *et al.*, 2008). The fruits of *Tridax procumbens* are hard achene. The structure partly aids the invasiveness or spread of *Tridax procumbens* from one location to the other. The seeds have pendulous embryos but lack endosperms. The calyx is mainly identified by scales or reduced pappus. The whole part of the plant is used to prevent and cure various ailments (Khan *et al.,* 2008).

Table 2.2. Names and languages of *Tridax procumbens*

Region/Language	Common Name	Reference(s)
Nigeria/Yoruba	Igbalode/Ewe Ehoro/Muwagun	Olowokudejo, 1987
Nigeria/Hausa	Harantama	Lewis, 1986
English	Coat buttons and Tridax daisy	Ankita and Jain, 2012
Latin	*Tridax procumbens* Linn.	Ankita and Jain, 2012
French	Herbe Caille	Ankita and Jain, 2012
Chinese	Kotobukigiku	Ankita and Jain, 2012
Japanese	Kotobukigiku	Holm *et al.*, 1997
El Salvador	Hierba del Toro	Holm *et al.*, 1997
Honduras	Hierba del Toro	Holm *et al.*, 1997
USA	Tridax daisy	Holm *et al.*, 1997
Ghana	Nantwi bini	Ravikumar *et al.*, 2005
India	Bisalyakarani, Vettukaya poondu	Komlaga *et al.*, 2015
Fiji	Wild Daisy	Komlaga *et al.*, 2015
Hawaii	Tridax	Komlaga *et al.*, 2015
Cuba	Romerillo de Loma, Romerillo	Komlaga *et al.*, 2015

2.3.4 Distribution and propagation of *Tridax procumbens*

Tridax procumbens is wild and distributed throughout the tropics. It is indigenous to tropical America and established in other regions such as Asia, Africa, Australia, and India. The plant is usually seen along roadsides, wastelands, and riverbanks, among others (Ankita and Jain, 2012). *Tridax procumbens* widespread results from its spreading stems and abundant seed production (Chauhan and Germination, 2008), and the propagation through seeds have produced variations. However, this plant cannot be micro-propagated via vegetative means such as cuttings and report has it that the herb's reproduction by seed requires exposure to the environment for growth and they are known to withstand adverse conditions such as drought (Ankita and Jain, 2012).

2.3.5 Physicochemical constituents of *Tridax procumbens*

Tridax procumbens has been reported to contain several phytochemical constituents, some of which are; tannin, terpenoids, triterpenes, luteolin, alkaloids, fumaric acid, quercetin, flavonoids, lauric acid, carotenoids, beta-sitosterol, oxoester, myristic, palmitic, arachidic acid, dexamethasone (Ara and Islam, 2020). Raju and Davidson, (1994) reported the presence of linolenic acid in the aerial parts of *Tridax procumbens* in addition to two characterised hydrophilic polysaccharides from its leaves.

Singh and Ahirvar, (2010) isolated and characterised a flavonoid (procumbensetin) from *Tridax procumbens*, and recently, Cui *et al.* (2020) isolated six bioactive compounds from *Tridax procumbens*. There are a few mineral constituents also in *Tridax procumbens* leaves namely; magnesium, sodium, calcium, potassium, and selenium. However, this plant is a valuable source of potassium supplements, plant protein, and provitamin A (carotenoids) (Chen *et al.*, 2008). Terpenoids and bis-bithiophene were isolated from *Tridax procumbens* (Ali and Jahangir, 2002).

Other constituents from *Tridax procumbens* are flavones in addition to puerarin, esculetin, and betulinic acid (Ankita and Jain, 2012). The compositions of the stem and leaf of Coat buttons were reported (Table 2.3), and the oleanolic acid derived from the plant was said to be an anti-diabetic agent (Verma and Gupta, 1998; Ali *et al.*, 2002). The flavones in *Tridax procumbens* have been said to possess antioxidant activity by evaluating their radical mopping activity (DPPH, FRAP) (Runsheg *et al.*, 2010).

Table 2.3. Proximate constituents of *Tridax procumbens* leaf and stem

	Stem		Leaf	
Indices	**W. Wgt**	**D. Wgt**	**W. Wgt**	**D. Wgt**
Energy (Kcal/100 g)	37.62 ± 0.61	321.54 ± 5.21	39.56 ± 0.26	397.59 ± 2.61
Water content	88.30 ± 0.02	Nil	90.05 ± 0.00	Nil
Lipid (%)	0.10 ± 0.01	0.85 ± 0.09	0.60 ± 0.02	6.03 ± 0.20
Protein (%)	4.38 ± 0.03	37.44 ± 0.26	3.44 ± 0.00	34.57 ± 0.00
Ash (%)	0.50 ± 0.01	4.27 ± 0.09	0.20 ± 0.02	2.01 ± 0.20
$C_x (H_2O)_y$ (%)	4.80 ± 0.01	41.03 ± 0.09	5.10 ± 0.02	51.26 ± 0.20
Fibre (%)	1.92 ± 0.03	16.41 ± 0.26	0.61 ± 0.04	6.13 ± 0.40

W. Wgt = Wet Weight, D. Wgt = Dry Weight

(Jude *et al.*, 2009)

2.3.6 Pharmacological activities and traditional uses of *Tridax procumbens*

2.3.6.1 Wound-healing effect

Bhat *et al.* (2007) reported that the juice from *Tridax procumbens* leaves has wound-healing effect by reducing wound contraction in experimented animals. This activity was compared with the activity of dexamethasone. It was discovered that *Tridax procumbens* on the contrary antagonises anti-epithelisation and tensile strength reducing the ability of dexamethasone.

In addition, this plant increased lysyl oxidase function and also enhanced wound healing in an experimental animal model. *Tridax procumbens* increased both protein and nucleic acid levels in granulation tissue, and this could be due to an elevation in the level of glycosaminoglycan (Nia *et al.,* 2003). The juice from *Tridax procumbens* has been utilised against bruises and wounds (Mahato and Chaudhary, 2005).

2.3.6.2 Hepatoprotective activity

D-galactosamine/lipopolysaccharide (d-GalN/LPS) reportedly caused the inflammatory condition of the liver and also destroys hepatocytes (Vilwanthan *et al.,* 2005). They cause multifocal necrosis in the liver which is similar to the lesion produced in viral hepatitis in humans. However, *Tridax procumbens* was reported to reduce these effects produced by d-GalN/LPS in the liver (Vilwanthan *et al.,* 2005).

2.3.6.3 Immunomodulatory effect

Tiwari *et al.* (2004) documented in their study the immunomodulatory effect of *Tridax procumbens* evidenced by elevation in some blood indices and activation of a humoral immune response.

2.3.6.4 Antidiabetic activity

A fraction from *Tridax procumbens* aqueous extract reportedly ameliorated elevated markers of diabetes in diabetic rats (Vyas *et al.,* 2004). Sonawane *et al.* (2014) documented its anti-diabetic activity

2.3.6.5 Antimicrobial activity

Tridax procumbens possess antimicrobial activity on different bacterial species using the disk diffusion method. Mahato and Chaudhary, (2005) have studied this method by using whole-plant extract on some microbes. The antimicrobial function was also assessed using this method on both fungal and bacterial strains (Manjamalai *et al.,* 2012). It was discovered that *E. coli*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* elicited more inhibition zones than S*treptococcus pneumonia*. Sathya *et al.* (2012) experimented with its antibacterial activity (*Staphylococcus aureus, Escherichia coli*, *Proteus mirabilis,* and *Vibrio cholera*) using different leaf extracts.

However, the antibacterial function of Coat buttons on *Staphylococcus aureus, Bacillus subtilis,* and *Enterobacter aerogenes* was assessed using ether, chloroform, hexane, and methanol extractions. The Agar well diffusion method was employed which concluded that the methanol extract contained a huge amount of bioactive compounds (Rizvi *et al.,* 2011). *Tridax procumbens* extracts exhibit toxicity against test fungi like phytopathogenic fungi (*Aspergillus niger*) and *Fusarium oxysporium* and also inhibit mycelial growth up to 60% (Bobbarala *et al.,* 2009; Jachak *et al.,* 2011). The 1.9 - 19.5 inhibitory concentration range was reported when *Tridax procumbens* was used against *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus* (Aniel and Naidu, 2011).

Yoga *et al.* (2009) also reported the antibacterial function of *Tridax procumbens* in food preservatives. However, both free and bound flavonoids from different components of the plant were identified for their antimicrobial activities and results show that *C. albicans* was the most susceptible organism. Kale and Dhake, (2013) also reported the action of hot water and methanol extracts of Coat button leaf on microbes. *Tridax procumbens* showed the highest activity against *S. typhi, S. flexneri* than against *E. coli* (Muthusamy *et al.,* 2013).

2.3.6.6 Antiparasitic activity

There are few reports of the antiparasitic potential of *Tridax procumbens*. However, Abubakar *et al.* (2012a) reported that it extracts possess insufficient antitrypanosomal activity.

2.3.6.7 Anti-inflammatory and analgesic activities

Awasthi *et al.* (2009) experimented with the role of *Tridax procumbens* leaf extract in inflammatory processes using carrageenan-mediated paw oedema in parallel with ibuprofen as a model. It was documented that the extract enhanced oedema inhibition after ibuprofen treatment. Meshram and Patil, (2011) also documented that *Tridax procumbens* extract suppresses inflammation in rat-paw oedema assay. The analgesic activity and reduction in abdominal writhing effects of *Tridax procumbens* were reported (Prabhu *et al.,* 2011).

2.3.6.8 Antioxidant activity

There is documentation of the *in vivo* antioxidant activities of *Tridax procumbens*. Singh *et al.* (2017) stated that *Tridax procumbens* ethanol extract possessed higher antioxidant functions than methanol and aqueous extracts by monitoring parameters such as DPPH. A similar observation was seen with the methanol extract of the aerial part via DPPH assay (Agrawal *et al.,* 2009). They concluded that ethyl acetate and n-butanol fractions possess a huge antioxidant property that is comparable to ascorbic acid as standard. Secondary metabolites characterised by their ethyl acetate fraction possess antioxidant activity (Saxena *et al.,* 2013)

2.3.6.9 Anticancer activity

There is a dearth of reports suggesting the anticancer action of the plant. However, the aqueous and acetone flower extract of *Tridax procumbens* was examined on the prostate epithelial cancerous cell line using the MTT test. The results thus suggest the *in vitro* anticancer property of the flower extract (Vishnu *et al.,* 2011).

2.3.6.10 Miscellaneous activities

Tridax procumbens has been utilised as herbal medicine against ailments such as jaundice, haemorrhage, and diarrhoea (Saraf and Dixit, 1991; Ali *et al.,* 2001). Ikewuchi, (2012) has investigated the role of *Tridax procumbens* aqueous leaf extract on haematology, serum biochemistry, and oxidative stress indices. The research on the antinociceptive activity of different doses of *Tridax procumbens* was reported (Ambulkar *et al.,* 2012). Brahma and Dhumal, (2014) reported the antiangiogenic property of alcoholic *Tridax procumbens* leaves extract.

Tridax procumbens has been used in several countries for different purposes. For example, blending the whole plant with other herbs has been documented to be used in some African countries for curing mastitis in livestock (Byavu *et al.*, 2000). Also in Guatemala, various parts of *Tridax procumbens* were said to be used in treating varying diseases and conditions such as colds, vaginitis, and mucosal inflammation hepatopathies (Pöll, 2005).

However, in Nigeria, it has been used in treating fever, cough, epilepsy, stomach ache, and Typhoid fever (Soladoye *et al.*, 2013). The Indians have extensively made use of the medicinal properties of this plant against diabetes, diarrhoea, hair loss, inflammation, jaundice, and wound healing (Saraf *et al.*, 1990; Pardeshi and Bhiungade, 2016).

CHAPTER THREE

METHODOLOGY

3.1 Protocol

3.1.1 Materials and chemicals

The chemicals and reagents used in this study such as Arsenic ($NaAsO_2$, Molecular weight 129.9, As 57.6%, CAS No 7784-46-5; Sigma) were of analytical grade. Other chemicals and reagents are listed in Table 3.1.

Table 3.1. Materials, equipment, chemicals, and reagents

Materials and Equipment	Chemicals and Reagents
Reagent bottles, beakers and measuring cylinders, lithium heparinised capillary tubes and sample tubes, sample collection bottles, digital weighing balance, petri dish with filter paper, slide, slide holder and slide rack, slide coverslip, desiccators, refrigerator, centrifuge, electric blenders, leukocyte reservoirs pipette, capillary pipettes, micropipettes, dissecting set, needle and syringes, microscope and tally counter, haemocytometer with cover glass, measurement ruler and timer, tissue paper, thread, cotton wool, hand gloves, stand with clamp, separating funnel, filter funnel, water bath, culture plates, pipette tips, class II biosafety cabinet, horizontal laminar flow workstation, mini gyro rocker, incubator, cylindrical chromatographic column, silica gel 60 (Mesh 230 - 400), UV light detector (254 nm), TLC plates (20 x 20 cm^2), TLC plate developing chamber, lead pencil, spatula, iodine chamber, cuvette.	May-Grὺnwald stain, Bouin solution, 10% formalin, diethyl ether, distilled water, MilliQ water, dimethyl sulfoxide, penicillin-streptomycin, blocking reagent, tween 20, trypsin EDTA, paraformaldehyde, BME, DMEM, MOPS/ EGTA/ magnesium sulphate/ formaldehyde, EDTA antigen retrieval, acetone, methanol, triton, foetal bovine serum, ethanol, dexamethasone, Minimum essential medium Eagle, bleach, phosphate buffer saline, hexane, dichloromethane, ethyl acetate, L-Glutamine solution, fluorescence mounting medium.

3.1.2 Isolation and characterisation of secondary metabolites in *Tridax procumbens*

3.1.2.1 Plant extraction

Tridax procumbens leaves were sourced from the University of Ibadan postgraduate hall garden, Nigeria. Identification was performed and a voucher with the number (UIH-22542) was kept in the Department of Botany herbarium (Appendix). *Tridax procumbens* fresh leaves (27 kg) were rinsed with clean tap water, air-dried, and ground into a fine powder (Plate 3.1). The 27 kg of fresh leaves yielded 5.5 kg (20.37%) of air-dried leaves which later yielded 3 kg (54.55%) of dried powdered leaves that were subjected to cold plant extraction as documented by Njar *et al.* (1993).

Cold absolute ethanol (96%) extraction (12 litres) was repeatedly carried out at an ambient temperature, and filtered using Whatman filter paper 1 (Sigma) (Figure 3.1). A rotary evaporator was utilised to concentrate the filtrate under reduced pressure at a stable temperature (40 °C). After concentration, 4.94% (148.258 g) extract (CETP) yield was obtained and preserved (at 4 °C) until when needed.

However, fractions, HXF, DCMF, EAF, and AQF (aqueous) were obtained when 80 g of CETP was fractionated in hexane, dichloromethane, and ethyl acetate, respectively using a separating funnel (500 ml). Briefly, the extract was dissolved in 200 ml ethanol: distilled H_2O (3:1) mixture, stirred, and the mixture emptied through a filter funnel into a separating funnel whose lid was closed. Thereafter, the funnel was removed from the stand, and inverted. The stopcock was opened to release the pressure in the funnel, the funnel was returned to the stand, and liquids were allowed to partition; yielding the corresponding soluble fractions.

The liquid at the lower layer of the funnel was carefully collected into a collecting beaker. This process was repeated for other liquids based on polarity i.e. HX ($CH_3CH_2CH_2CH_2CH_2CH_3$) < DCM (CH_2Cl_2) < EA (CH_3-C(=O)-O-CH_2-CH_3). 2.4L, 1.8L, and 1.8L of hexane, dichloromethane, and ethyl acetate, respectively were utilised during the separation. The separation yield was 37.87 g (47.34%), 8.46 g (10.58%), 2.23 g (2.79%), and 19.97 g (24.96%) for HXF, DCMF, EAF, and AQF (residuals), respectively. *Tridax procumbens* fractions collected were concentrated

using a water bath (40 °C) while the concentrates were then preserved at 4 °C until when needed for use.

3.1.2.2 Column and Thin Layer Chromatography (TLC)

Chromatographic techniques play a huge role in plant products chemistry and the discovery of novel and potent bioactive compounds relevant in the industry and medicine (Bajpai *et al.,* 2016). The procedure was done in line with a previous study (Bajpai *et al.,* 2016). Briefly, 1 g of powdered dichloromethane fraction of *Tridax procumbens* was dissolved in dichloromethane solvent. The solution was pre-absorbed in silica gel (3 - 5 g), mixed, and dried in a rotary evaporator to a powdered form. Silica gel (50 g) was dissolved in dichloromethane solvent to form a slurry and loaded into a cylindrical column.

The side of the column was rinsed properly to remove any silica. The column was repeatedly tapped to allow the silica to sediment, well compacted, and the top almost dried. The tap of the column was opened to remove the remaining solvent. The dried sample was poured on top of the silica in the column. Thereafter, 50 ml of dichloromethane solvent with a gradient of methanol (0 - 20%, v/v) was eluted through the column at a constant rate under gravity to partition the test sample. The fractionated samples were collected in a test tube and labelled appropriately for further investigation using thin-layer chromatography (Figure 3.2). A spot of the sample was made on an activated TLC plate using a capillary tube at a 1cm distance from the plate's bottom edge. The plate was inserted in a chamber having a 10% methanol in methanol/dichloromethane mixture (v/v) until the solvent mixture almost reached the plate's upper edge. The plate was removed, air-dried, and the solvent front demarcated. Bands were visualised using UV light (254 nm, 365 nm) in the iodine compartment.

A spray reagent (vanillin-sulphuric acid) was also applied to identify definite compounds. The calculated retention factor (R*f*) of the individual band is = distance covered by the spot (cm)/distance covered by the solvent (cm). R*f* of about 0.2 difference between bands showed good separation and was used. Dichloromethane with a gradient of methanol samples showing a similar pattern of separation on TLC was pulled together [0 - 3%, D1; 4 - 5%, D2; 6 - 7%, D3; and 8 - 20%, D4; methanol/dichloromethane mixture (v/v)].

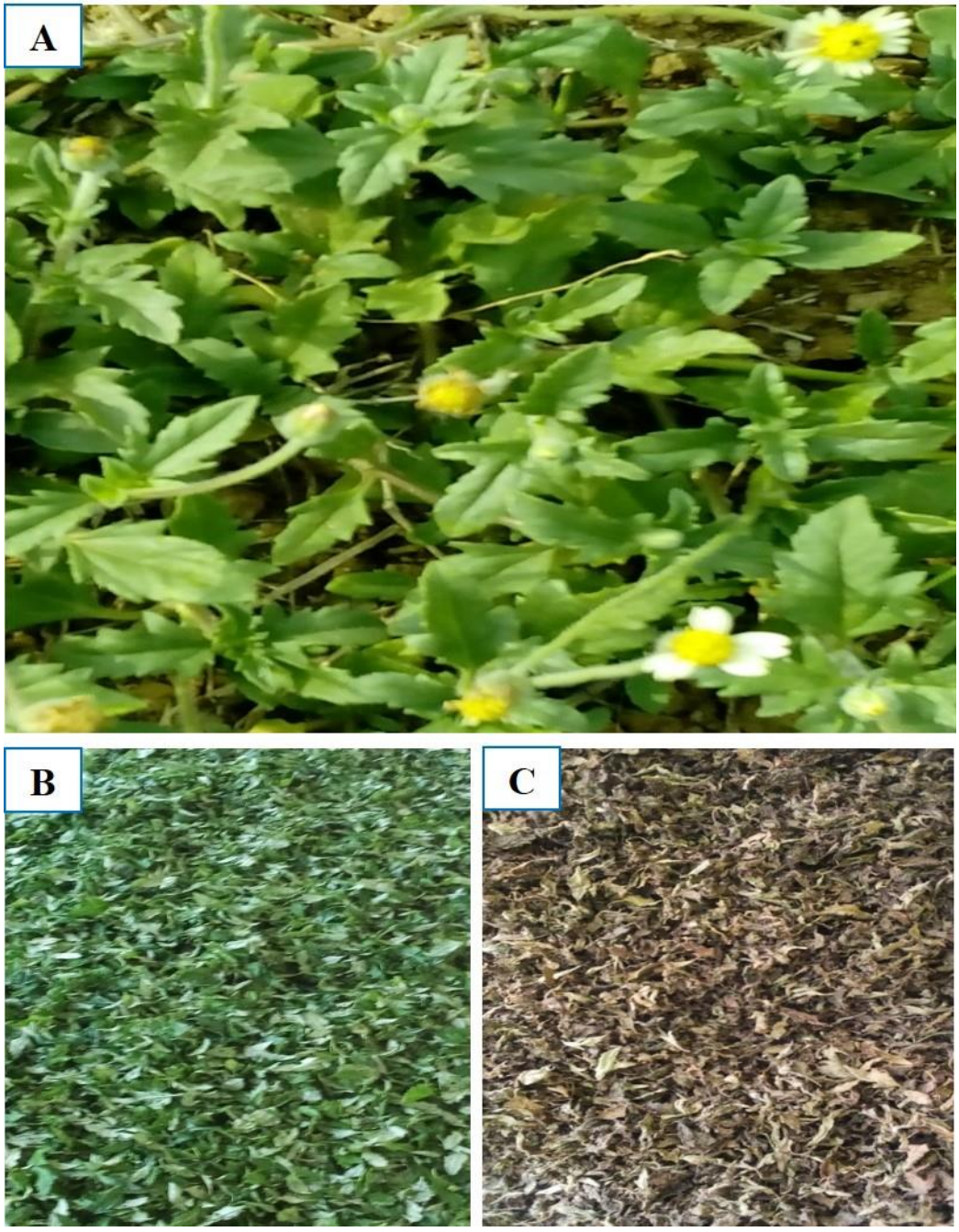


Plate 3.1. *Tridax procumbens* herb (A: branches with leaves and flowers, B: freshly washed leaves, C: air-dried leaves). 9:14:12am, 4th March 2015, Postgraduate hall garden, University of Ibadan.

Figure 3.1. The extraction process of *Tridax procumbens* ethanol leaf extract. Extraction was carried out at the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan.

3.1.2.3 LC-MS and LC-Tandem MS

The most potent fraction was analysed by utilising UHPLC/QTOF MS (Bruker, Germany). This was carried out in both electrospray plus- and minus-ion MS manner. This QTOF was linked to an UltiMate 3000 UHPLC (Thermo Fisher). Nebulizing gas, drying gas, and capillary voltage was set to 4 bar, 12 L/min and 4500V, respectively with a 75-1500 m/z time-of-flight scan range at 220 °C.

Tandem MS collision energy was placed on a scale of 100, 500 and 1000 m/z at 14.0, 20.0, and 30.0 eV, respectively. The liquid chromatographic separation was done utilising a column (C18 (2), 100 Å, 4.6 mm x 250, 5 micrometers, Phenomenex); 1 mL/min flow rate, 35°C, 10 µL volume of injection. The mobile phases A and B consisted of H_2O and C_2H_3N/CH_3CN, respectively, and both with 0.1% v/v HCOOH. Gradient timely elution was performed with 5% mobile B in a continuous gradient to 95% B at 20 minutes, maintained 95% B up until 25 minutes, eventually moved back to 5% B up until 28 minutes' complete procedure time.

The mass spectrometer was standardised by utilising an array of HCOONA clusters introduced by changing valve injection in the first minute of an individual run. Three parts of 1 M Na hydroxide, ninety-seven parts of 50:50 H_2O-C_3H_8O with 0.2% HCOOH were employed for the mass calibrant solution. 90 mg of the subfraction of dichloromethane fraction of *Tridax procumbens* was used as a start sample to isolate five bioactive compounds.

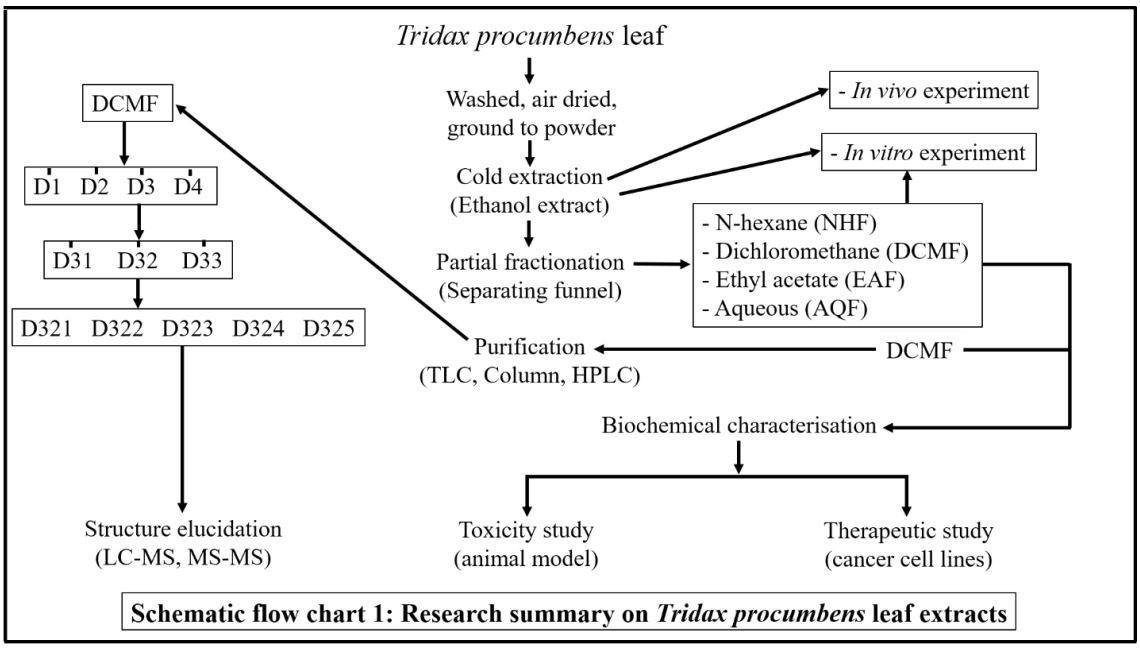


Figure 3.2. A systematic approach used in the characterisation of bioactive compounds from *Tridax procumbens* leaf.

3.1.3 Experimental animal ethical considerations

The University's Ethics Committee (UI-ACUREC) approved this project with reference no: UI-ACUREC/App/12/2016/03 (Appendix) for all relevant protocols and procedures used. This conforms to standard guidelines for laboratory animal care (NIH, 1985). The Universities Accreditation Scheme at the University of Bath, UK also approved some aspects of this project with reference no; 42787 (Appendix) according to the Animal (Scientific Procedures) Act, 1986.

80 - 100 g body weight (bwt.) 32 male Wistar rats were obtained (animal house, Faculty of Veterinary Medicine). The rats were stabilised and observed (one week) before being used. The healthy rats were accommodated in steel laboratory cages. The rats were maintained under 12-hour dark and light cycles each with temperature and relative humidity of 25 ± 2 °C and 50 ± 15% respectively.

The animals had unrestricted access to water and commercial diet (Vital Feed Nig., Ibadan®). Prepared sample suspensions (5 mg/100 g body weight/0.1 ml olive oil) to be administered were maintained at room temperature. The thirty-two experimental rats were distributed equally into four (n = 8) with the treatment modalities as follows:

Group A - 1 ml/kg bwt. olive oil (1:1 v/v) (negative control) daily for 14 days
Group B - 2.5 mg/kg Sodium Arsenite (SA) (positive control) twice (7th and 14th day)
Group C - 50 mg/kg Crude Extract of *Tridax procumbens* (CETP) daily for 14 days
Group D - 50 mg/kg CETP daily for 14 days and 2.5 mg/kg SA on days 7 and 14
(Preston *et al.*, 1987, Abubakar *et al.*, 2012b).

The oral route of administration was used for all the treatments. 24 hours post the last treatment; aliquots of blood were obtained from the rats after which they were euthanised by cervical dislocation under mild diethyl ether anaesthetics.

3.2 *In vitro* studies and protocols

3.2.1 Phytochemicals examination

The extract and fractions of *Tridax procumbens* were screened for plant metabolites as reported (Trease and Evans, 1989; Sofowora, 1993).

3.2.1.1 Anthraquinones

The extract and fractions of *Tridax procumbens* (0.5 g) were boiled in a water bath with 10 ml of sulphuric acid (H_2SO_4). The filtrate was mixed with chloroform (5 ml), and allowed to stand for 5 minutes. The lower chloroform layer was decanted into a new tube and mixed with diluted ammonia (1 ml). The solution was observed for any colour changes. The appearance of pink to red colour of the ammoniacal layer indicated anthraquinone presence.

3.2.1.2 Terpenoids (Salkowski test)

In a test tube containing the extract and fractions of *Tridax procumbens* (0.5 g), chloroform (2 ml) was added and undiluted H_2SO_4 (3 ml) was also added along the side. A reddish brown colour at the junction signifies the terpenoids' presence.

3.2.1.3 Steroids (Liebermann-Burchard test)

100 mg each of the extract and fractions of *Tridax procumbens* was added and shaken in a test tube containing chloroform (2 ml). Afterwards, drops of acetic anhydride were included and the mixture was boiled in a water bath. This was left alone to cool in iced water and undiluted H_2SO_4 (2 mL) was included along the side. The advent of a brown ring at the boundary of 2 strata, together with the top stratum turning green indicated the steroids presence.

3.2.1.4 Cardiac glycosides

Glacial acetic acid (2 ml) that contains a drop of $FeCl_3$ solution followed by undiluted H_2SO_4 (1 ml) was mixed with 5 ml *Tridax procumbens* extract and fractions (100 mg/ml). The advent of brown rings at the junction signified cardiac glycoside. Violet and greenish rings were formed below and above the brown ring, respectively. The green ring which is located in the acetic acid zone slowly spreads across the zone.

3.2.1.5 Saponins

5 ml of distilled water was vigorously mixed with *Tridax procumbens* extract and fractions (0.5 g) in a tube. The formation of persisting frothing on warming for 5 minutes indicates the presence of saponins.

3.2.1.6 Tannins

10 ml of distilled water was mixed with *Tridax procumbens* extract and fractions (0.5 g) in a tube, and boiled. Drops of 0.1% $FeCl_3$ were mixed with the filtrate, and the appearance of brown-green or blue-black colour was indicative of tannins.

3.2.1.7 Alkaloids

10 ml of *Tridax procumbens* crude extract and fractions (0.25 mg/ml) in 1% HCL was heated with a water bath (5 minutes) and filtered. This filtrate was split into two parts. To one part was added 1 ml of Dragendorff reagent (BiI_4K). The formation of an orange-red precipitate was indicative of alkaloids. For the other part, 1 ml Mayer's reagent ($K_2[HgI_4]$) was mixed with a cream-coloured precipitate indicative of alkaloids.

3.2.1.8 Total phenol content

The procedure was determined as reported (Singleton and Rossi, 1965) by utilising the Folin ciocalteu's phenol reagent as electron acceptor. Each 0.1 ml of test substance (0.1, 0.08, 0.06, 0.04, 0.02 mg/ml) and distilled H_2O (0.9 ml) was mixed with the oxidising agent (0.2 ml), and the mixture vortexed. 1.0 ml of 7% Na_2CO_3 (w/v) was thereafter included after 5 minutes of standing, and the mixture was distilled to 2.5 ml before incubation (90 minutes, 25 ^oC). Absorbance over a negative control (1 ml distilled H_2O replaced the sample) was then obtained (750 nm). 0.1 mg/ml Gallic acid was employed as the standard for Gallic Acid Equivalent (GAE) of test substance determination and distilled H_2O was utilised as blank.

3.2.1.9 Total flavonoid content

This experiment was performed in line with the $AlCl_3$ colorimetric assay technique (Miliauskas *et al.*, 2004). Quercetin (standard) with varied amounts (100, 200, 300, 400, 500 μg/ml) was employed. To 0.1 ml extract and fractions of *Tridax procumbens* and quercetin were added distilled water (0.4 ml). Subsequently, 0.1 ml of 5% $NaNO_2$

were included and while waiting for 5 minutes, 10% $AlCl_3$ (0.1 ml) and NaOH (0.2 ml) was included and the volume scaled up (2.5 ml). Absorbance at 510 nm was assessed over the blank. Calculated total flavonoid content (mg quercetin equivalents/gram (mgQUE/g) of test samples) was derived:

$$T = C\ x\ \frac{VL}{WT} \text{ ----------3.1}$$

where T = total flavonoid content in quercetin equivalent, C = derived quercetin concentration via the standard curve, VL = volume of the test sample (ml), WT = obtained test sample weight

3.2.2 Plant antioxidants

3.2.2.1 Total antioxidant capacity

The ability of a substance to convert Molybdenum VI compound to Mo (V). The consequent appearance of a measurable green PO_4^{-3}/Mo (V) colouration forms the basis of this test (Prieto *et al.*, 1998). 1 ml of 3/5 M sulphuric acid, 28 mM Na_3PO_4, and 4 mM NH_4^+ molybdate mixture combined with 0.1 ml test samples [*Tridax procumbens* extract, vitamin C (0.02, 004, 0.06, 0.08, and 0.1 mg/ml)], were incubated (95 ^oC, 90 minutes). This was left to stand and cooled to 25 ^oC. Absorbance over a negative control (distilled water replaced the sample) was then obtained (695 nm) and the antioxidant activities of the samples were evaluated as the standard equivalent (AAE).

3.2.2.2 2,2-diphenyl-1-picrylhydrazyl hydrate

Tridax procumbens extract and fractions metabolite scavenging activity was determined as documented (Brand-Williams *et al.*, 1995). This radical was reduced after reacting with a compound that possesses the ability to donate hydrogen (Blois, 1958). This resulted in a deep colour change to light yellow that was thereafter determined (517 nm). In summary, 0.3 mM DPPH (1 ml) in CH_3OH was combined with 1 ml of *Tridax procumbens* extract/fractions, and the standard (vitamin C) (10, 5, 2.5, 1.25, 0.6 25, 0.3125 mg/ml). The solution was stirred and incubated without light (30 minutes). Absorbance was determined at 517 nm over control (1 ml methanol replaced the sample).

The percentage of inhibition was measured as:

Percentage inhibition = $[(A_c-A_s)/A_c] \times 100$ ----------3.2

Control absorbance (reagent minus sample) = A_c, extract absorbance = A_s. IC_{50} was derived (graph of % inhibition/sample concentration).

3.2.2.3 Nitric oxide radical

The inhibition or scavenging property of *Tridax procumbens* extract and fractions on nitric oxide radical (NO) was carried out as reported (Marcocci *et al.*, 1994). NO radical obtained from Na nitroprusside at normal pH conditions in an aqueous mixture interacts with O_2 to form NO_2 ions (measured by Griess reaction).

This mixture comprising 0.1 ml (10, 5, 2.5, 1.25, 0.625, 0.3125 mg/ml) extract or control (ascorbic acid), 0.9 ml $C_5FeN_6Na_2O$ (2.5 mM) in PO_4^{3-} buffer was incubated (25 ^oC, 150 minutes) and 0.5 ml of 1% $C_6H_8N_2O_2S$ in 5% H_3PO_4 was included and also incubated (10 minutes, without light). 0.5 ml 0.1% $C_{12}H_{14}N_2.2ClH$ was later included. A chromophore appeared and the absorbance was obtained (546 nm).

Percentage inhibition = $[(A_c-A_s)/A_c] \times 100$ ----------3.3

Control absorbance (reagent minus sample) = A_c, extract absorbance = A_s. IC_{50} was derived (graph of % inhibition/sample concentration).

3.2.2.4 Ferric reducing antioxidant power (FRAP)

Antioxidants and spectrophotometers are employed as reducing agents and to determine absorbances, respectively in this procedure (Benzie and Strain, 1999). This relies on ferric-tripyridyltriazine (colourless) reduction to its ferrous state (blue).

50 µl each of *Tridax procumbens* extract/fractions (0.1 mg/ml), and vitamin C (20, 40, 60, 80, and 100 μg/ml) were combined with FRAP reagent (1 ml). 593 nm absorbance taken 10 minutes after mixing over the blank (50 µl distilled H_2O). Measurements were obtained in the dark (room temperature) and FRAP was evaluated as equivalent concentration [antioxidant concentration that produced a ferric reducing ability similar to vitamin C (AAE)].

3.3 *In vivo* assays

3.3.1 Haematology

An aliquot of blood was obtained through the retro-orbital sinus into sterile lithium heparinised tubes from rats that were lightly anaesthetised with diethyl ether. The animal was gently scruffed, and removed from a desiccator, and the eye was allowed to bulge. The lithium heparinised capillary pipette was inserted medially and the blood was allowed to flow via capillary action into the capillary tubes.

Haematocrit (PCV), red blood corpuscles (RBC) counts, haemoglobin concentration (HB), white blood corpuscles (WBC) (total and differential) counts, and platelet (PLT) counts were analysed (Abbott Cell Dyn). In addition, RBC, HB and PCV values were then utilised to estimate MCV, MCH, and MCHC (Ikewuchi and Ikewuchi, 2013) as stated below.

$$MCV = \frac{10 \times \text{haematocrit (\%)}}{\text{RBC } (\times 10^6/\mu l)} \text{ (fL)} \quad \text{----------3.4}$$

$$MCH = \frac{10 \times \text{Hb (g/dl)}}{\text{RBC } (\times 10^6/\mu l)} \text{ (pg)} \quad \text{----------3.5}$$

$$MCHC = \frac{100 \times \text{Hb (g/dl)}}{\text{haematocrit (\%)}} \text{ (g/dl)} \quad \text{----------3.6}$$

3.3.2 Serum chemistry

An aliquot of blood was placed into sterile sample tubes without an anticoagulant. The blood sample, on standing, clotted and was centrifuged (3000 rpm, 10 minutes). Supernatant from this was obtained and the concentrations of total protein, albumin, bilirubin, cholesterol, lipoproteins (high HDL; low LDL; very low VLDL), triglyceride, sodium, potassium, calcium, and inorganic phosphate were assessed, while AST, ALP, GGT, and ALT activities were monitored with Randox kit (UK) and analysed (ATAC 8000, Elan Diagnostics, CA, USA).

3.3.2.1 Total protein

Estimation of total protein concentration was done as described (Tietz, 1995). Cupric ions in a basic medium interact with peptide bonds leading to coloured complex formation.

Reagent 1 (I ml) was combined with 0.02 ml of each of the control, standard, and sample in a cuvette under room temperature. This was mixed, and incubated (30 minutes, 20 - 25 ^{o}C), and absorbances (sample A_s and standard A_{std}) were measured over control (blank). Total protein concentrations were measured at 546 nm as:

Total protein concentration = 190 x A_s (g/dl), or = (A_s/A_{std}) x standard concentration (when using a standard) ----------3.7

3.3.2.2 Albumin

Estimation of serum albumin concentration was done as described (Tietz, 1987b). This estimation is based on the principle that albumin binds quantitatively to 3,3',5,5'-tetrabromo-m-cresol sulphonephthalein. The albumin B-C-G complex absorbs maximally (578 nm) and absorbance equates to the sample's albumin level.

BCG Reagent 1 (3 ml) was combined with 0.01 ml of each of the control, standard, and sample in a test tube under room temperature. This was mixed, and incubated (30 minutes, 20 - 25 ^{o}C), and absorbances (sample A_s and standard A_{std}) were measured over control (blank) using a spectrophotometer (600 - 650 nm).

This was mixed, and incubated (30 minutes, 20 - 25 ^{o}C), and absorbances (sample A_s and standard A_{std}) were measured over control (blank). The albumin level of the sample was estimated as

Albumin concentration (g/l or g/dl) = (A_s/A_{std}) x standard conc. ----------3.8

3.3.2.3 Gamma glutamyl transferase (GGT)

GGT in the test serum converts L-y-glutamyl-3-carboxy-4-nitroanilide to 5-amino-2-nitrobenzoate in the presence of glycylglycine (Tietz, 1987a) that was estimated (405 nm).

Reagent 1 (1 ml) was joined with 0.1 ml of each sample in a cuvette (25 ^{o}C, 30 ^{o}C, 37 ^{o}C). The solution was mixed and absorbance was read at times 0, 1, 2, and 3 minutes using a spectrophotometer (405 nm). GGT was estimated thus:

U/L = 1158 x ▲A405 nm/min ----------3.9

3.3.2.4 Alkaline phosphatase (ALP)

Alkaline phosphatase in the test sample converts p-nitrophenyl phosphate to phosphate and p-nitrophenol (Rec GSCC, 1972). Reagent 1 (macro, 3 ml; semi-micro, 1 ml; micro, 0.5 ml) was mixed with 0.05, 0.02, and 0.01 ml, respectively, of each sample in a cuvette (25 ^{o}C, 30 ^{o}C, 37 ^{o}C). The solution was mixed and absorbance was read at times 0, 1, 2, and 3 minutes using a spectrophotometer (405 nm). ALP was estimated thus: U/L = 3300 x ▲A405 nm/min (macro); U/L = 2760 x ▲A405 nm/min (semi-micro, micro) ----------3.10

3.3.2.5 Aspartate aminotransferase (AST)

Aspartate aminotransferase in the test sample catalyses the reaction of α-oxoglutarate and L-aspartate into L-glutamate and oxaloacetate. Oxaloacetate hydrazone produced with 2,4-dinitrophenylhydrazine was used to monitor the activity of AST (Schmidt and Schmidt, 1963).

Reagent 1 (0.5 ml) was combined with 0.1 ml of each of the control and sample in a test tube. This was mixed, incubated (30 minutes, 37 ^{o}C), and Reagent 2 (0.5 ml) was added and allowed to stand for 20 minutes (20 - 25 ^{o}C). Afterwards, sodium hydroxide (5 ml) was mixed and the absorbance of the sample after 5 minutes was read over the control (blank) using a spectrophotometer (546 nm).

The activity of serum AST was estimated from a given absorbance (Abs) and U/I values

Abs	U/I	Abs	U/I
0.02	7.0	0.1	36.0
0.03	10.0	0.11	41.0
0.04	13.0	0.12	47.0
0.05	16.0	0.13	52.0
0.06	19.0	0.14	59.0
0.07	23.0	0.15	67.0
0.08	27.0	0.16	76.0
0.09	31.0	0.17	89.0

3.3.2.6 Alanine aminotransferase (ALT)

Alanine aminotransferase in the test sample converts α-oxoglutarate and L-alanine into L-glutamate and pyruvate. Pyruvate hydrazine produced with 2,4-dinitrophenylhydrazine was used to monitor the activity of ALT (Schmidt and Schmidt, 1963).

Reagent 1 (0.5 ml) was combined with 0.1 ml of each of the control and sample. This was mixed, incubated (30 minutes, 37 ^{o}C), and Reagent 2 (0.5 ml) was added and allowed to stand for 20 minutes (20 - 25 ^{o}C). Afterwards, sodium hydroxide (5 ml) was mixed and the absorbance of the sample after 5 minutes was read over the control (blank) using a spectrophotometer (546 nm). The activity of serum ALT was estimated from a given absorbance (Abs) and U/I values

Abs	U/I	Abs	U/I
0.250	4.0	0.275	48.0
0.050	8.0	0.300	52.0
0.075	12.0	0.325	57.0
0.100	17.0	0.350	62.0
0.125	21.0	0.375	67.0
0.150	25.0	0.400	72.0
0.175	29.0	0.425	77.0
0.200	33.0	0.450	83.0
0.225	39.0	0.475	88.0
0.250	43.0	0.500	94.0

3.3.2.7 Total Bilirubin

Total serum bilirubin level was monitored in the presence of dimethylsulphoxide by the interaction with diazotized sulphanilic acid (Tietz, 1990). Reagent 1 (1 ml) was mixed with 200 µl of each sample and standard in a test tube. This was mixed and left alone for 5 minutes (20 - 25 ^{o}C), and the absorbances of the sample and standard were read over the control (blank) using a spectrophotometer (546 nm).

The total bilirubin of the sample was estimated as = $(A_{sample}/A_{standard})$ x standard concentration ----------3.11

3.3.2.8 Creatinine

Creatinine level was determined according to Tietz, (1987a). This protein reacts with picric acid to elicit a coloured complex whose amount is equivalent to the creatinine level. Working reagent (macro, 2 ml; micro, 1 ml) was combined with the corresponding 0.2 ml each (macro) and 0.1 ml (micro), respectively of standard and sample in a test cuvette (25 ^{o}C, 30 ^{o}C, 37 ^{o}C).

The solution was mixed and absorbance was read at times 0, 1, 2, and 3 minutes. The solution was mixed and 30 secs later, absorbance (A1) (standard, sample) was obtained. In another 2 minutes, absorbance (A2) (standard, sample) was obtained using a spectrophotometer (490 - 510 nm).

Creatinine level was calculated: A2 - A1 = ▲A $_{sample}$ or ▲$A_{standard}$.

Therefore, Creatinine (mg/dl) = (▲A_{sample}/▲$A_{standard}$) x 2 ----------3.12

3.3.2.9 Blood urea nitrogen

Ammonia was obtained from the hydration reaction of serum urea in the presence of urease. This NH_3 was later evaluated photometrically using Berthelot's reaction (Tietz, 1987a). Urea + water $\xrightarrow{\text{Urease}}$ $2NH_3 + CO_2$

NH_3 + hypochlorite + phenol ⟶ indophenol (blue colouration) ----------3.13

Reagent 1 (10 µl) was combined with 10 µl each of the control, sample, and standard in a test tube. This was mixed and incubated for 10 minutes (37 ^{o}C). Reagent 2 and Reagent 3 (2.5 ml each) were correspondingly added instantly and incubated for 15 minutes (37 ^{o}C). Absorbances of the sample (A_{sample}) and standard ($A_{standard}$) were read over control (blank) using a spectrophotometer (546 nm) while the reaction colour was stable for a minimum of 8 hours.

Blood urea nitrogen (mg/dl) = A_{sample}/ $A_{standard}$) x standard concentration----------3.14

3.3.2.10 Cholesterol

Cholesterol level was monitored after hydrolysis and oxidation reactions. Quinoneimine was formed from H_2O_2 and 4-aminoantipyrine while utilising phenol and peroxidase. Reagent 1 (1000 µl) was combined with 10 µl each of distilled H_2O (control), sample, and standard in a cuvette. This was mixed and incubated for 10

minutes (20 - 25 ^{o}C) or 5 minutes (37 ^{o}C), and the absorbance (sample) was obtained over the control within 60 minutes using a spectrophotometer (500 nm).

Cholesterol level (mg/dl) = A_{sample}/ $A_{standard}$) x standard concentration ----------3.15

3.3.2.11 Triglycerides

Triglycerides level was monitored after hydrolysis and oxidation reactions. Quinoneimine was formed from H_2O_2, 4-aminoantipyrine and 4-chlorophenol while utilising peroxidase.

Reagent 1 (1000 µl) was mixed with 10 µl each of the sample and standard in a cuvette. This was mixed and incubated for 10 minutes (20 – 25 ^{o}C) or for 5 minutes (37 ^{o}C), and the absorbance (sample) was obtained over the control within 60 minutes using a spectrophotometer (500 nm).

Triglycerides level (mg/dl) = A_{sample}/ $A_{standard}$) x 200 ----------3.16

3.3.2.12 HDL-Cholesterol

Phosphotungstic acid in the presence of Mg ions causes lipoproteins to be precipitated in the serum. After centrifugation, the cholesterol level in the remaining HDL fraction of the supernatant was assessed. Reagent 1 (1000 µl) was combined with 100 µl each of distilled H_2O (control), sample, and standard in a test tube. This was mixed and incubated for 10 minutes (20 - 25 ^{o}C) or for 5 minutes (37 ^{o}C), and the absorbance (sample) was obtained over the control within 60 minutes using a spectrophotometer (500 nm).

HDL cholesterol level (mg/dl) = A_{sample}/ $A_{standard}$) x standard concentration ---------3.17

LDL cholesterol was derived from HDL cholesterol level as below

LDL = Total cholesterol - triglyceride/5 - HDL (mg/dl) ----------3.18

3.3.2.13 Serum sodium

Serum sodium was assessed with slight modification (Trinder, 1951; Maruna, 1958). Here, sodium was released as a triple salt; sodium $C_8H_{12}MgO_{10}U$, and the extra uranium reacted with ferrocyanide that produced a colour. The absorbance of the chromophore varied inversely to the test sample Na concentration. Test tubes (blank (distilled H_2O), standard, sample) were labelled and filtrate reagents (1.0 ml) were pipetted into them. 50 µl each of the sample and distilled water was included in the

corresponding tube and homogenised. These were vigorously shaken (3 minutes), centrifuged at 1500 G (10 minutes), and the supernatant was examined as follows; acid reagent (1 ml) was transferred into another set of test tubes as above. A supernatant (50 µl) was mixed with the solution and later 50 µl of colour reagent was mixed with it. Absorbance (sample) was obtained by utilising a spectrophotometer (550 nm).

$$\frac{(A_{blank} - A_{sample})}{(A_{blank} - A_{standard})} \text{ x concentration of standard (mEq/L) = concentration of sample (mEq/L)} \quad \text{----------3.19}$$

3.3.2.14 Serum calcium

Ca ions will form a violet complex with O-Cresolphthalein complexone in a basic medium. 0.5 ml each of solutions 2 and 3 was added to 25 µl each of distilled water (control), sample and standard (solution 1) in a test tube. This was mixed, incubated (20 - 25 ^{o}C/37 ^{o}C), and absorbance (sample, standard) was obtained over the control after 5 to 50 minutes using a spectrophotometer (550 - 590 nm).

Calcium level (mg/dl) = $A_{sample}/ A_{standard}$) x 10 = (mg/dl) ----------3.20

3.3.2.15 Serum inorganic phosphorus

Serum inorganic phosphorus interacts with NH_4 molybdate while utilizing H_2SO_4. A complex of phosphomolybdate was formed and monitored. Reagent 1 (1000 µl) was combined with 10 µl each of distilled H_2O (control), sample and standard in a test tube.

This was mixed and incubated for 10 minutes (20 - 25^{o}C) or 5 minutes (37 ^{o}C), and the absorbance (sample, standard) was obtained over the control using a spectrophotometer (340 nm).

$$\frac{A_{sample}}{A_{standard}} \text{ x concentration of standard = inorganic phosphorus (mq/dL)}$$

mg/dl x 10/30 = mg/dl x 0.3333 = mM/L, 3.4 x 0.3333 = 1.13mM/L ----------3.21

3.3.3 Histological analysis

Liver, kidney, brain, lung, and testes were excised from the animals after sacrifice, perfused with chilled 1.15% KCl, blot-dried, weighed, and measured. These tissues were stored in 10% formalin solution except for the testes that were stored in bouin solution. Subsequent tissue sectioning and histological examination were done

(Chayen *et al.*, 1973). These tissues were fixed in a 10% formalin solution to preserve the tissue morphology and chemical composition. The tissues were dehydrated in graded concentrations of ethanol ranging from 70% - 100% and cleared in xylene to impregnate the tissue with paraffin solvent. Infiltration was done in 50% v/v xylene and ethanol for two hours and embedded in molten paraffin wax and retained in the oven at 56 - 58 ^{o}C to allow for penetration of paraffin wax into the intercellular spaces of the tissues to facilitate sectioning.

Small blocks of paraffin containing the tissues were sectioned using the blade of the microtome adjusted for the 5 mm-thick paraffin sections. Mounting and staining were done when the sections were placed in warm water before being transferred to clean slides and stained with hematoxylin and counter-stained with eosin in a specific order (immersed in absolute alcohol, 70% ethanol, water and hematoxylin, water, and 70% ethanol again, then eosin, 80% ethanol and lastly absolute ethanol). The prepared slides were examined and photomicrographs were taken by employing a light microscope and an Ortholux microscope fitted with a Leitz camera unit, respectively. Analysis was carried out subsequently.

3.3.4 Reproductive studies

The experimental animals were mildly anaesthetised with diethyl ether and euthanised through cervical dislocation. For sample collection, a prescrotal cut was made and the testicles were expressed outside of the site to expose them. An aliquot of semen was collected thereafter from the caudal epididymis and the samples were then analysed immediately for gross motility, live/dead ratio, and morphology of the spermatozoa after the collection as described by Zemjanis (1970).

3.3.4.1 Sperm volume

Sperm volume was assessed using a calibrated measuring cylinder after semen collection.

3.3.4.2 Sperm count and motility

Spermatozoa motility and count were determined as described (Zemjanis,1970; Pant and Srivastava, 2003). Spermatozoa were numbered using a haemocytometer and an improved Neubauer chamber (LABART, Germany).

3.3.4.3 Morphological derangement and percentage live/dead ratio

Four hundred spermatozoa in total from individual rats were assessed for morphological changes in smears derived from Wells and Awa stains [1/5 g and 3/5 g of Eosin and Fast green, respectively dispersed in distilled H_2O and C_2H_5OH mixture (2:1)]. The percentage viability was calculated by utilising a 1% Eosin and 5% Nigrosin in a 3% $Na_3C_6H_5O_7.2H_2O$ mixture as reported (Wells and Awa, 1970).

3.3.5 Genotoxicity: micronucleus assay

In vivo micronucleus assay is usually utilised mainly for assessing chemicals for their chromosome-breaking effects (Thayer, 2003). The strength of this test is in its ability to estimate DNA injury and repair in cells (proliferating, non-proliferating) (Butler, 2005). In this study, a bone marrow micronucleus test was employed as reported (Schmid, 1975) with slight modifications.

Briefly, the animals were euthanised and the femur was removed. A sized needle was introduced into the marrow opening via the epiphyseal extremity of the femur to extrude the marrow from the iliac end. The extruded tissue was spread onto a glass slide containing 1 - 2 fetal calf serum drops and homogenised into a fine cell suspension and smeared using a clean edge of another slide. The slides were air-dried, and fixed in CH_3OH for 5 minutes, air-dried again, and stained in May-Grùnwald Stain 1 (0.4 % May-Grùnwald stain: dissolved 0.4 g of May-Grùnwald stain in absolute methanol and made up with absolute methanol to 100 mL) for 3 - 5 minutes, Stain 2 (1:1 (v/v) dilution of Stain 1 with distilled water) for another 3 - 5 minutes, and rinsed.

Lastly, the slides were air-dried, mounted in DPX mountant with coverslips, and scored using a tally counter under a light microscope (x100 objective lens) to detect polychromatic erythrocytes (PCEs) and micronuclei. The PCEs and micronuclei are stained blue and normal mature erythrocytes was stained red. The frequencies of micronuclei in the PCEs (mPCEs) were then determined by scoring 1000 PCEs per animal.

3.3.6 Cell culture experiment

3.3.6.1 Human Panc-1 cells and HepG2 cells

Human cell lines (Panc-1, HepG2) were donated by Professor David Tosh, Biology and Biochemistry Department, University of Bath, UK. The cell lines were sourced from ECACC, a culture collection of public health England, United Kingdom. The cell culture study was done in the laboratory of Professor David Tosh using a BioMAT Class II Biosafety Cabinet. Briefly, a cryovial containing frozen cell suspension at -80 ^{o}C was inserted in a water bath to defrost for 1 - 2 minutes (37 °C). The cryovial was decontaminated with 70% ethanol while the defrosted cells in the vial immediately moved into a sterile centrifuge tube containing culture medium; DMEM fortified with 1/10th FBS, penicillin (10 units/0.1 mL), streptomycin (0.1 mg/mL) (Sigma).

The suspension was centrifuged (Thermo Electron Corporation, France) for 3 minutes (1000 rpm) and the supernatant was pipetted while they were subsequently remixed in 1 ml DMEM. The resuspended cells were then seeded into T75 cm^2 culture flasks containing 10 ml DMEM and incubated (5% CO_2, 95% atmospheric air) at 37 ^{o}C. The DMEM was changed 24 hours after the seeding, thereafter, every other day until the cells reached a confluence of 75 - 80% or when cells needed to be split. Subculture (1:3 - 1:5) was done every 7±1 days. The cells were thereafter frozen, preserved in FBS containing 1/10th (v/v) dimethyl sulfoxide (DMSO), and stored in liquid N_2 or at -80 ^{o}C until when needed.

3.3.6.2 Cell counting

Cell counting for seeding and analysis was done using a haemocytometer. Briefly, DMEM was removed from wells containing adherent cells and washed with 1x phosphate buffer saline (PBS) for 1 - 2 minutes. These cells were then dissociated by trypsinisation with 0.05% Trypsin EDTA (Gibco) for 5 - 10 minutes in an incubator at 37 ^{o}C. Complete trypsinisation was confirmed by viewing the cells under the microscope and was stopped by adding DMEM.

The mixture was aspirated into a test tube, centrifuged (3 minutes, 1000 rpm), supernatant pipetted, and the centrifuged cells remixed in 1 ml DMEM. The same amount of monolayer suspension was mixed with trypan blue dye in an Eppendorf

tube, mixed, and placed on the haemocytometer ready for counting. These cells were scored and the concentration for seeding was calculated thus with the formula;

Cell concentration/ml = TN x D x 10^4 ----------3.22

Where TN is the average sum of cells scored in 4 squares, D is the dilution factor (total volume of mixture used/ volume of cell suspension aliquot), 10^4 (0.1 mm^3 = 1 x 10^{-4}).

3.3.6.3 Cytotoxicity assays

3.3.6.3.1 Trypan Blue Exclusion

The test was carried out in adherence to instruction (ThermoFisher) and based on the principle that trypan blue dye passes through the compromised cell membrane of dead or dying cells. This will allow the dye to stain the nucleic acid blue making it trypan blue positive. However, live cells exclude the dye and remain bright and translucent under a light microscope, therefore making them trypan blue negative.

Human Panc-1 cells and HepG2 cells were plated at 10 x10^2 cells/well on a coverslip (Fisher) in a 4-well plate, incubated (24 hours), and exposed to varying amounts (0.01, 0.02, 0.05, and 0.1 mg/ml) of *Tridax procumbens* extract and fractions for additional 24 and 48 hours. After treatment, the culture medium was evacuated by pipetting and washed with PBS for 1 - 2 minutes. The cells were then trypsinised with Trypsin EDTA (200 µL) for 3 - 5 minutes in an incubator. Trypsinisation was confirmed by viewing the cells with a microscope (Tension) and stopped by adding DMEM (800 µL).

The mixture was centrifuged (1000 rpm in 3 minutes) and the supernatant was decanted after which, the cells were remixed in 1 ml DMEM. 20 µL aliquot of resuspended cells included to 20 µL of Trypan blue dye (1:1) for 1 - 3 minutes. The mixture was placed on a haemocytometer and examined immediately under the light microscope at low magnification to detect blue staining cells and scored the total cells number. Four squares were viewed and the cells were counted with a haemocytometer. The relative number of viable cells was measured:

% positive cells = [1- (viable cells/ total cell number)] x 100 ----------3.23

3.3.6.3.2 MTT {3-(4,5-dimethylyhiazol-2-yl)-2,5-diphenyl tetrazolium bromide}

Reagents/materials (microplate reader measuring absorbance aD 590 - 600 nm (Modulus Microplate, Turner BioSystems Inc), pipettes and pipette tips, tubes for reagents and buffer solutions preparation, 96-well plate, foil, MTT reagent (50 mL), MTT solvent (150 mL). The MTT test was employed to measure and quantify cell multiplication, viability, and cell toxicity.

This assay relied on yellow-soluble tetrazolium salt compound cleavage to a non-water-soluble purple formazan by mitochondrial succinate dehydrogenase. Living cells will usually convert MTT into formazan products. The formazan's concentration will then be determined by optical density at a given absorbance. The measured absorbance is equivalent to the number of live cells. However, this ability is lost in dead cells hence, they will show no signal. The assay was performed as described (Abcam, UK). Panc-1 cells were plated (1 $x10^4$ cell/well) in a ninety-six-well plate, incubated (24 hours), and treated with varying concentrations (20, 50, 100, and 250 μg/ml) of *Tridax procumbens* samples for additional 24 hours. DMSO concentration of less than 0.5% in DMSO-exposed cells was utilised.

3.3.6.3.3 Live/Dead

Reagents [calcein acetoxymethyl (CAM) (20 nM in anhydrous dimethyl sulfoxide) and ethidium homodimer-1 (EHD-1) (2 nM in DMSO/H_2O 1:4 (v/v) dyes]. A Live/dead cytotoxicity kit (Invitrogen) was employed to assess the presence of live and dead cells cultured on different coverslip biomaterial surfaces by using both dyes.

The live/dead test relied on the principle that live and dead cells retain calcein and EHD-1, respectively. The live cells are noticed due to the activity of intracellular esterase that catalyses the transformation of a cell-permeant CAM (non-fluorescence) to polyanionic dye calcein (intensely fluorescent). This dye is trapped within viable cells and produces a bright evenly green fluorochrome (~495/~515 nm). EHD-1 permeates cells with compromised membranes and binds to nucleic acids. This binding will thereby produce a forty-fold enhancement of mainly bright fluorescence (red) in non-viable cells (~495/~635 nm). Usually, EHD-1 is repelled by an uncompromised viable cell membrane. The assessment of viability then depends on these cell characteristics.

3.3.6.3.3.1 *Fluorescent microscopy procedure*

Human Panc-1 and HepG2 cells were incubated (37 ^{o}C, 5% CO_2, 24 hours) after passaging at 10 x104 cells/well in a 4-well plate and exposed to varying levels (20, 50, and 100 μg/ml) of *Tridax procumbens* samples dissolved in DMSO for additional 24 and 48 hours. A final DMSO level of less than 0.5% in DMSO-treated cells were utilised. Following cells rinsing with PBS solution two times, they were incubated with 0.4 ml working solution that comprised 20 µM CAM (1:200) and 2 µM EHD-1 (1:5000) in PBS for 25 - 30 minutes at 25 ^{o}C (in the dark). Following incubation, cells were rinsed subsequently with 1x PBS. Fluorescence of the live (green) and dead cells (red) were viewed under a fluorescence microscope (Leica, DMI 4000B) and a Confocal Laser Scanning Microscope (LSM 880). Three randomly selected regions were focused and cells were counted. The percentage number of viable and dead cells and their mean intensities were determined utilising Image J software.

3.3.6.3.3.2 *Flow cytometry procedure*

Human Panc-1 cells were incubated (37 ^{o}C, 5% CO_2, 24 hours) following passaging at 5 x10^5 cells/well in a 6-well plate and exposed to 20 μg/ml of *Tridax procumbens* extracts dissolved in DMSO for another 24 hours. DMSO-treated cells were employed as control (final concentration is 0.2%) following administration, cells were rinsed with PBS solution two times and trypsinised (Trypsin EDTA) for 3 - 5 minutes in an incubator.

Trypsinisation was halted following DMEM addition. The mixture was centrifuged (1000 rpm in 3 minutes), supernatant pipetted, and the cells remixed in 1 ml working solution that comprised 20 µM CAM (1:200) and 2 µM EHD-1 (1:5000) in PBS. The cells were incubated (15 - 20 minutes, 25 ^{o}C, in the dark), and thereafter analysed using the flow cytometry method (488 nm excitation) as described (BD Biosciences, USA). Green (viable cells) and red (dead cells) fluorescence emissions for calcein (530/30 bandpass) and EHD-1 (610/20 bandpass), respectively were measured after gating on cells to exclude the debris.

3.3.7 Isolation and Culture of Mouse Embryonic Organs

3.3.7.1 Pancreas, Intestine, Oesophagus, and Liver

Pancreatic, intestinal, oesophagus, and liver tissues were dissected from a mouse embryo at E11.5d of development according to the method described by Ward and Tosh (2010). This was carried out with the use of a fine dissecting instrument; jeweller's forceps, sharp scissors; fine tungsten needle (Sigma) in an H-MAT horizontal laminar flow workstation. Briefly, a pregnant female mouse was euthanised by cervical dislocation and the uterus-containing embryo was removed and placed in a 35 mm culture dish (Nalgene Nunc, Thermo Fisher) having an autoclave phosphate buffer saline (9.55 g/l in MilliQ dH_2O).

The uterus was rinsed in dissecting media, Minimum Essential Medium Eagle (MEM) that comprised fortified Hank's salts (1/10th FBS; 1% L-glutamine, 200 mM; 0.2% gentamicin, 0.01 mg/ml), and the outer protective covering excised with the use of dissecting forceps and sharp scissors to release the embryo. The embryos were then decapitated and the pancreas, intestine, oesophagus, and liver were isolated while viewing under a dissecting microscope (Leica m28, m275).

Cylinder cloning rings (SLS, UK) were placed on a coverslip (11 - 12 mm diameter) coated with 50 µl bovine plasma fibronectin (1000 μg fibronectin was dissolved in 1 ml of MilliQ dH_2O, stored at -20 ^oC) (Invitrogen) in a cell culture multiwell plate (1.9 cm^2 culture area). 0.75 - 1 ml culture media (BME); fortified Earle's salts (1/10th FBS; 1% L-glutamine, 200 mM; 0.2% gentamicin, 0.01 mg/ml) was then added. The isolated organs were transferred with the use of the Gilson p200 pipette into the cloning ring and incubated (24 hours, 37 ^oC, 5% CO_2). The explant was cultured for five days to allow for the proper development of the organs with their distinct normal phenotypes. The tissues were treated with the extract and different fractions of *Tridax procumbens* (20 µg/ml) in DMSO as the control for an additional two days.

3.3.8 Immunofluorescence staining

Immunofluorescence was done as reported (Shen *et al.*, 2003). Treated cultured cells or dissected embryonic organs were fixed using fixatives like; 4% paraformaldehyde (PFA), acetone: methanol (Ac: Me) or MOPS/EGTA/Magnesium sulfate/4% Formaldehyde (MEMFA (10% formaldehyde; 0.1 M MOPS; 2 mM EGTA 1 mM

$MgSO_4$, pH 7.4) in buffer saline (Sigma, St Louis, Mo), depending on the antibody to be used. However, fixation of dissected embryonic tissues is generally performed in MEMFA for 15-20 minutes (room temperature) because it provides a suitable condition for most tissues, especially the liver and pancreatic biomarkers. On the other hand, cultured cells do well in 4% PFA at 4 ^{o}C for 15 - 20 minutes. However, most Ac: Me (1:1) at -20 ^{o}C fixed cells and tissues usually take 5 minutes.

After fixation, rinse coverslips three times for 5 minutes each in phosphate buffer saline. Permeabilisation was done using 1% Triton X-100 (tissues) and 0.1% Triton X-100 (cells) (Sigma) in phosphate buffer saline for 15 - 20 minutes (not necessary for Ac: Me fixation) and rinsing gently several times in buffer saline before leaving at 4 ^{o}C until when needed. Antigen retrieval was done using EDTA on those tissues or cells fixed in Ac: Me for 1 - 2 hours or 30 - 45 minutes, respectively at 37 ^{o}C in an incubator.

The embryonic tissues or cells were blocked to discourage non-specific binding of antigen with the use of two per cent buffer (50,000 mg, blocking reagent dissolved in 100 mM maleic acid, 150 mM sodium chloride, pH 7.5 in PBS, 1 - 2 hours) (commercially available blocking buffer was used). Primary antibodies mixed in a two per cent blocking buffer in PBS and incubated overnight (4 ^{o}C) were used to probe the tissues or cells. Secondary antibodies diluted in a two percent blocking buffer in PBS were used after washing off any unbounded primary antibodies three times for five minutes each.

The tissues or cells were then incubated (2 - 3 hours, 25 ^{o}C) and rinsed in PBS 3 times for 5 minutes each. Coverslips were mounted on well-labelled slides (VWR) utilising fluorescence mounting medium (Dako), and DAPI as nuclei stain (Invitrogen). The slides were viewed afterwards (Leica, DMI 4000B, confocal microscope (LSM 880)).

Table 3.2. Primary and secondary antibodies

Primary antibody				Secondary antibody (Life Technologies)	
Antibody	Species	Company	Dilution	Antibody	Dilution
Alpha Fetoprotein	Rabbit	Dako	2:200	Alexa Fluor® 594 chicken anti-rabbit Texas Red goat anti-rabbit	2:800 2:200
Albumin	Rabbit	Sigma	2:200	Alexa Fluor® 594 chicken anti-rabbit	2:800
Transferrin	Rabbit	Dako	2:200	Alexa Fluor® 594 chicken anti-rabbit	2:800
Alpha1-antitrypsin	Goat	Sigma	2:200	Alexa Fluor® 488 chicken anti-goat	2:800
Sox9	Rabbit	Millipore	2:200	Alexa Fluor® 594 chicken anti-rabbit	2:2000
β-catenin	Mouse	Sigma	2:200	Alexa Fluor® 488 goat anti-mouse	2:500
GST pi	Mouse	BD	2:200	Fluorescein horse anti-mouse	2:200
ALP	Goat	Sigma	2:200	Texas Red rabbit anti-goat	2:200
Caspase 3	Mouse	Sigma	2:200	Fluorescein horse anti-mouse	2:200
Amylase	Rabbit	Sigma	1:200	Texas Red goat anti-rabbit	2:200
APC	Rabbit	Spring Bioscience	2:200	Texas Red goat anti-rabbit	2:200
Vimentin	Mouse, Goat	Sigma	2:200	Fluorescein horse anti-mouse, Fluorescein rabbit anti-goat	2:200 2:200
Cytokeratin 7	Mouse	Sigma	2:200	Texas Red horse anti-mouse	2:200
PNA	Biotinylated	Vector Lab	2:200	AMCA Streptavidin	2:200
Catalase	Rabbit	Rockland	2:200	Fluorescein horse anti-mouse	2:200
Cdx2	Goat	Biogenex	2:200	Texas Red rabbit anti-goat	2:200
Ki-67	Mouse	BD	2:200	Texas Red horse anti-mouse	2:200
CAII	Sheep	The Binding Site	2:200	Fluorescein rabbit anti-sheep	2:200
Bcl-2	Mouse	Dako	2:200	Fluorescein horse anti-mouse	2:200
Insulin	Guinea pig	Dako	1:500	Alexa Fluor® 594 chicken anti-guinea pig	1:500
E-cadherin	Mouse	BD	2:200	Fluorescein goat anti-rabbit	2:200
Glucagon	Mouse	Sigma	2:200	AMCA horse anti-mouse	2:200
Somatostatin	Rabbit	Dako	2:200	Fluorescein goat anti-rabbit	2:200
p53	Mouse	Abcam	1:300	Texas Red horse anti-mouse	2:200
$p21^{Cip1/Wap-1}$	Rabbit	Santa Crux Biotechnology	2:200	Alexa Fluor® 488 chicken anti-rabbit	2:500

3.4 Statistical analysis

Results were analysed (T-test, ANOVA, Dunnett post hoc). Graph Pad Prism (4.0) and Microsoft Corporation (2010) were utilised while images collected with microscopes were processed (Image J, Zen 2.3 lite). Values were presented as mean ± SEM at $\alpha_{0.05}$.

CHAPTER FOUR

RESULTS

4.1 Objective 1: Phytochemical constituents of *Tridax procumbens* ethanol leaf extract

Flavonoids, terpenoids, tannins, steroids, alkaloids, saponins, anthraquinone, and phenols were seen to be present following the qualitative phytochemical examination of *Tridax procumbens* extract (Table 4.1).

Table 4.1. Qualitative phytochemicals of *Tridax procumbens* extract

Phytochemicals	CETP
Saponins	++
Tannins	+
Flavonoids	+
Anthraquinone	++
Cardiac glycoside	-
Terpenoids	+
Steroids	++
Alkaloids	+
Phenols	+

+ = Present

++ = More abundant

- = Absent

CETP: crude extract of *Tridax procumbens*. n = 3

4.2. Objective 2: *Tridax procumbens* leaf extract modulates dysfunctions mediated by sodium arsenite in male Wistar rats

It was observed that the animals' weights in all the groups increased throughout the experiment and their percentage body weight changes were not significant as compared to the control (Figure 4.1) except for the significant reduction in body weight change of the group treated with SA. Also, the relative organ weights for the groups (Table 4.2) were not significant.

The total relative kidney weight of the extract-only exposed group reduced significantly relative to the control and SA-only exposed group (Table 4.3). However, the mean value of the relative liver and brain weights of the group exposed to SA increased significantly relative to the group given extract only, and SA co-exposed with extract, respectively (Table 4.2). Total relative testicular weights for the group treated with SA reduced significantly in relation to the extract-only treated group (Table 4.9).

The reduction in the HB and RBC values of groups treated with SA only and SA co-administered with extract was significant in comparison to the control. Also, the PCV value in the SA co-administered with extract group was reduced significantly relative to the control (Table 4.4). The changes in the mean values of MCV, MCH, MCHC, lymphocytes, neutrophils, monocytes, and eosinophil were not significant for all the groups (Table 4.4). The extract at 50 mg/kg could not ameliorate the haemotoxicity mediated by arsenite in the test rats.

Similarly, the values for WBC in rats of groups treated with SA only and SA co-exposed with extract were significantly elevated relative to the control. Also, the platelet counts in SA co-exposed with the extract group were reduced significantly relative to the control (Table 4.4). The platelet counts of the group treated with extract only were non-significantly elevated relative to the control (Table 4.4). The variations in the values of cholesterol, triglyceride, HDL, LDL, and VLDL among the groups were non-significant in comparison to the control (Table 4.5). The mean values for the levels of sodium, potassium, inorganic phosphate, and calcium in all the groups were not different significantly. But the level of inorganic phosphate in the group treated with SA only and SA co-administered with extract was reduced significantly (Table

4.6). The amount of total protein, albumin, globulin, bilirubin, creatinine, BUN, and activity of ALP among the groups did not significantly change (Table 4.7). However, a notable elevation in the activity of AST in the SA-only treated rats, and both AST and ALT in the extract only exposed rats in comparison to control (Figure 4.2) was observed.

Non-significant alterations in values for the volume of spermatozoa and spermatozoa viability (Figure 4.3) were observed among the groups. Moreover, a significant reduction in spermatozoa motility and concentration values was noticed (groups B, C, and D). The mean values for the sperm cell morphology of all the groups did not change significantly. Significant alterations in the values of the bent tail and curved tail of the groups treated with SA only and the extract only were observed in comparison to the control (Table 4.8). Meanwhile, no noticeable significant change was observed in testicular and epididymal morphometry among the groups (Tables 4.9 and 4.10).

The arsenite-induced micronuclei formation (35 folds) significantly in rat bone marrow cells compared to the control and other test groups (Figure 4.4). The *Tridax procumbens* extract ameliorated this toxic effect to approximately 6 folds in the co-exposed group (Figure 4.4). The histology of the liver, lungs, kidney, testes, cerebellum and cerebrum were examined (Plate 4.1 – 4.6). It was seen that sodium arsenite caused alterations in the architecture of the organs monitored (Plate 4.1 – 4.6). The extract was able to ameliorate these alterations except for the similar lesions noticed in the liver of the extract, and arsenite-treated rats (Plate 4.5).

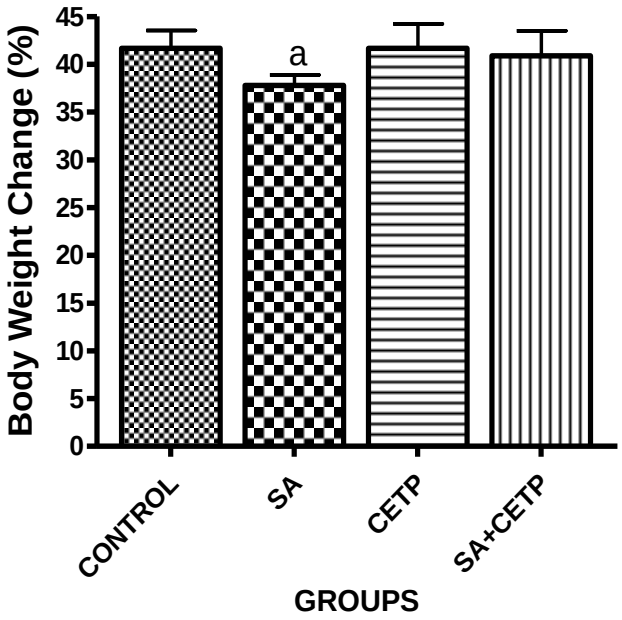


Figure 4.1. Percentage of body weight change of experimental rats in treated groups.

[a] significant ($p < 0.05$) from control. Olive oil (Control), sodium arsenite (SA) and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. n = 8.

Table 4.2. Relative organ weights of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS (%)	A	B	C	D
Liver	3.78 ± 0.246	4.34 ± 0.186[a]	3.80 ± 0.055[a]	3.94 ± 0.242
Brain	0.962 ± 0.068	1.12 ± 0.049[a]	0.982 ± 0.055	0.956 ± 0.043[a]
Lungs	0.820 ± 0.042[a]	0.802 ± 0.045[b]	0.768 ± 0.078[c]	0.812 ± 0.091[d]
Heart	0.352 ± 0.015[a]	0.386 ± 0.012[b]	0.374 ± 0.022[c]	0.378 ± 0.019[d]
Spleen	0.534 ± 0.118[a]	0.372 ± 0.037[b]	0.428 ± 0.048[c]	0.464 ± 0.071[d]

Each result is mean ± SEM (n = 8). Results with similar superscripts [a-d] within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.3. Renal morphometry of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS	A	B	C	D
Rel. right kidney weight (%)	0.346 ± 0.012	0.352 ± 0.007[a]	0.286 ± 0.024[a]	0.286 ± 0.024[a]
Rel. left kidney weight (%)	0.350 ± 0.013[a]	0.324 ± 0.008	0.294 ± 0.016[a]	0.316 ± 0.017
Total rel. kidney weight (%)	0.696 ± 0.023[a]	0.676 ± 0.014[b]	0.580 ± 0.034[ab]	0.644 ± 0.026
Right kidney diameter (cm)	2.72 ± 0.124[a]	2.30 ± 0.084[a]	2.50 ± 0.089	2.58 ± 0.132
Left kidney diameter (cm)	2.66 ± 0.169[a]	2.06 ± 0.306[b]	2.60 ± 0.078[c]	2.44 ± 0.075[d]
Total kidney diameter (cm)	5.38 ± 0.271[a]	4.36 ± 0.333[a]	5.10 ± 0.155	5.02 ± 0.162
Right kidney length (cm)	1.40 ± 0.063	1.24 ± 0.093[a]	1.34 ± 0.040[b]	1.54 ± 0.040[ab]
Left kidney length (cm)	1.38 ± 0.037[a]	1.32 ± 0.066[b]	1.44 ± 0.144[c]	1.42 ± 0.066[d]
Total kidney length (cm)	2.78 ± 0.058	2.56 ± 0.129[a]	2.78 ± 0.159	2.96 ± 0.087[a]

Each result is mean ± SEM (n = 8). Results with similar superscripts [a-d] within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.4. Haematological parameters of test animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS	A	B	C	D
PCV (%)	45.8 ± 0.66^{a}	43.0 ± 1.05^{a}	50.0 ± 3.02^{b}	41.2 ± 0.66^{a}
HB (g/dl)	15.3 ± 0.296^{ab}	14.3 ± 0.317^{a}	14.5 ± 0.46	13.5 ± 0.233^{b}
RBC ($x10^{12}$/l)	7.66 ± 0.116^{ab}	7.09 ± 0.207^{a}	7.12 ± 0.174	6.90 ± 0.185^{b}
MCV (f l)	59.8 ± 0.641^{a}	60.7 ± 0.496^{b}	61.0 ± 0.877^{c}	59.8 ± 0.731^{d}
MCH (pg)	20.0 ± 0.239	20.1 ± 0.176	20.3 ± 0.200^{a}	19.6 ± 0.221^{a}
MCHC (g/dl)	33.4 ± 0.180^{a}	33.2 ± 0.335	33.3 ± 0.379	32.7 ± 0.1
Platelets ($x10^{11}$µg)	1.15 ± 0.056^{a}	1.32 ± 0.072	1.29 ± 0.157	0.96 ± 0.040^{a}
WBC ($x10^{9}$/µg)	2.90 ± 0.430^{a}	4.57 ± 0.189^{ab}	4.09 ± 0.467	3.59 ± 0.155^{b}
Lymphocytes (%)	64.6 ± 2.16	70.6 ± 1.66^{a}	66.6 ± 2.58	59.0 ± 2.77^{a}
Neutrophils (%)	31.8 ± 2.27	26.0 ± 1.76^{a}	30.6 ± 2.73	37.8 ± 3.17^{a}
Monocytes (%)	1.80 ± 0.490^{a}	1.60 ± 0.245^{b}	1.40 ± 0.245^{c}	1.60 ± 0.245^{d}
Eosinophils (%)	1.80 ± 0.374^{a}	1.80 ± 0.374^{b}	1.40 ± 0.678^{c}	1.60 ± 0.510^{d}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.5. Lipid profile of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS (mg/dl)	A	B	C	D
Cholesterol	46.0 ± 3.05^{a}	51.4 ± 2.23^{a}	50.0 ± 3.02^{b}	49.8 ± 4.65^{c}
Triglyceride	60.6 ± 3.37^{a}	66.0 ± 1.67^{b}	63.6 ± 3.78^{c}	66.6 ± 2.50^{d}
HDL	16.0 ± 1.52^{a}	17.0 ± 1.55^{b}	17.6 ± 1.47^{c}	19.6 ± 3.04^{d}
LDL	17.9 ± 2.15^{a}	21.2 ± 0.91^{b}	$19.7 \pm 1.15^{c\ c}$	16.9 ± 1.82^{d}
VLDL	12.1 ± 0.674^{a}	13.2 ± 0.335^{b}	12.7 ± 0.755^{c}	13.3 ± 0.500^{d}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.6. Electrolytes parameters of test animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS	A	B	C	D
Sodium (mEq/L)	146 ± 1.03^{a}	144 ± 1.89^{b}	144 ± 2.20^{c}	145 ± 1.93^{d}
Potassium (mEq/L)	6.06 ± 0.254^{a}	5.98 ± 0.222^{b}	6.42 ± 0.462^{c}	6.12 ± 0.340^{d}
Phosphorus (mg/dl)	6.78 ± 0.086^{ab}	5.78 ± 0.260^{a}	6.20 ± 0.182	5.72 ± 0.348^{b}
Calcium (mg/dl)	12.1 ± 0.311^{a}	11.8 ± 0.186^{b}	11.6 ± 0.509^{c}	11.5 ± 0.235^{d}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.7. Serum chemistry of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS	A	B	C	D
Total protein (g/dl)	7.14 ± 0.385^{a}	7.20 ± 0.300^{b}	7.26 ± 0.383^{c}	7.36 ± 0.407^{d}
Albumin (g/dl)	3.08 ± 0.334^{a}	3.12 ± 0.275^{b}	3.20 ± 0.276^{c}	3.32 ± 0.275^{d}
Globulin (g/dl)	4.06 ± 0.093^{a}	4.08 ± 0.049^{b}	4.06 ± 0.121^{c}	4.04 ± 0.163^{d}
Albumin-Globulin Ratio	0.758 ± 0.075^{a}	0.766 ± 0.063^{b}	0.782 ± 0.048^{c}	0.820 ± 0.054^{d}
Total bilirubin (mg/dl)	0.340 ± 0.051^{a}	0.360 ± 0.051^{b}	0.440 ± 0.068^{c}	0.280 ± 0.066^{d}
Blood urea nitrogen (mg/dl)	17.4 ± 0.350^{a}	17.2 ± 0.344^{b}	17.3 ± 0.371^{c}	17.4 ± 0.246^{d}
Creatinine (mg/dl)	0.780 ± 0.066^{a}	0.840 ± 0.093^{b}	0.800 ± 0.055^{c}	0.860 ± 0.108^{d}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

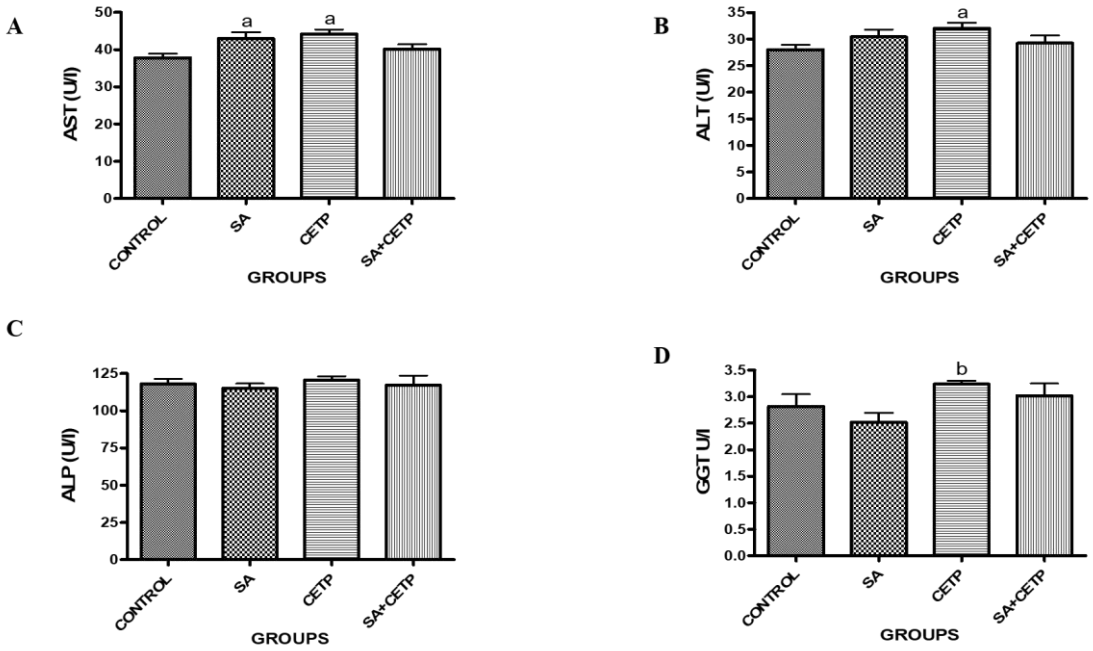


Figure 4.2. Liver damage biomarkers of experimental rats in treated groups.

[a and b] significant ($p < 0.05$) from control and arsenite, respectively. Olive oil (Control), Sodium arsenite (SA) and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. n = 8.

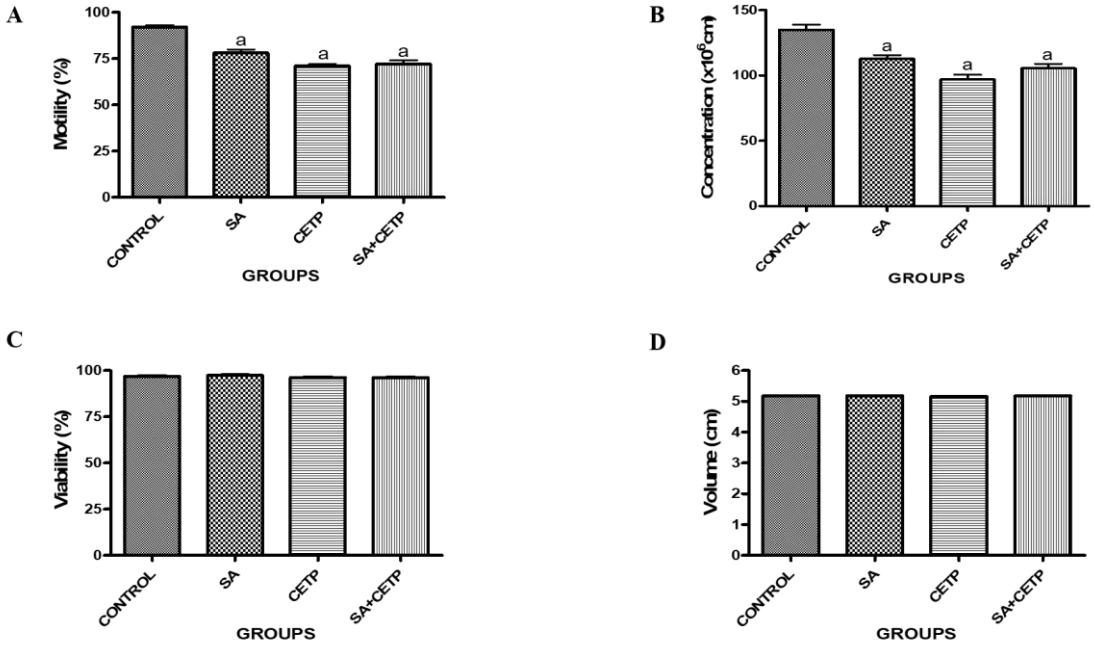


Figure 4.3. Spermatozoa characterisation of rats in treated groups.

[a] significant ($p < 0.05$) from control. Olive oil (Control), Sodium arsenite (SA) and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. n = 8.

Table 4.8. Sperm morphology of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS (%)	A	B	C	D
Tailless head	1.13 ± 0.123^{a}	1.13 ± 0.123^{b}	1.14 ± 0.128^{c}	1.33 ± 0.060^{d}
Headless tail	1.14 ± 0.129^{a}	1.03 ± 0.142^{b}	1.04 ± 0.145^{c}	1.04 ± 0.146^{d}
Rudimentary tail	0.49 ± 0.110^{a}	0.49 ± 0.112^{b}	0.54 ± 0.094^{c}	0.55 ± 0.093^{d}
Bent tail	2.52 ± 0.104^{ab}	1.97 ± 0.108^{acd}	3.02 ± 0.160^{bd}	2.57 ± 0.195^{c}
Curved tail	2.23 ± 0.178^{ab}	2.07 ± 0.128^{acd}	2.82 ± 0.174^{bd}	2.58 ± 0.066^{c}
Curved mid-piece	2.37 ± 0.175^{a}	2.07 ± 0.092^{abc}	2.63 ± 0.109^{c}	2.38 ± 0.244^{d}
Bent mid-piece	2.47 ± 0.080^{a}	2.07 ± 0.092^{abc}	2.72 ± 0.110^{c}	2.67 ± 0.132^{b}
Looped tail	0.54 ± 0.090^{a}	0.49 ± 0.112^{b}	0.54 ± 0.094^{c}	0.49 ± 0.111^{d}
Total abnormal cells	12.89	11.47	14.45	13.61
Total normal cells	87.11	88.53	85.55	86.39

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.9. Testicular morphometry of experimental animals treated with *Tridax procumbens* and/or sodium arsenite

PARAMETERS	A	B	C	D
Left testis length (cm)	2.02 ± 0.049^{a}	2.06 ± 0.209^{b}	2.46 ± 0.051^{a}	2.20 ± 0.114^{c}
Left testis diameter (cm)	2.82 ± 0.213^{a}	3.02 ± 0.020^{b}	2.70 ± 0.110^{c}	2.78 ± 0.086^{d}
Right testis length (cm)	2.06 ± 0.025^{a}	2.02 ± 0.020^{b}	2.46 ± 0.051^{ab}	2.34 ± 0.154^{c}
Right testis diameter (cm)	2.96 ± 0.051^{a}	3.06 ± 0.252^{b}	2.88 ± 0.058^{c}	2.96 ± 0.051^{d}
Rel. right testis wgt (g)	0.612 ± 0.062^{a}	0.536 ± 0.039^{b}	0.668 ± 0.044^{b}	0.118 ± 0.016^{d}
Rel. left testis wgt (g)	0.606 ± 0.060^{a}	0.514 ± 0.036^{b}	0.678 ± 0.048^{b}	0.614 ± 0.075^{c}
Rel. total testis wgt (g)	1.22 ± 0.121^{a}	1.05 ± 0.074^{b}	1.35 ± 0.091^{b}	1.22 ± 0.144^{c}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

Table 4.10. **Epididymal morphometry of experimental animals treated with *Tridax procumbens* and/or sodium arsenite**

PARAMETERS	A	B	C	D
Left epididymal length (cm)	3.66 ± 0.186^{a}	3.34 ± 0.189^{b}	3.64 ± 0.280^{c}	3.66 ± 0.254^{d}
Left epididymal diameter (cm)	1.42 ± 0.136^{a}	1.56 ± 0.183^{b}	1.54 ± 0.169^{c}	1.68 ± 0.086^{d}
Right epididymal length (cm)	3.10 ± 0.134^{a}	3.22 ± 0.080^{b}	3.48 ± 0.146^{c}	3.14 ± 0.181^{d}
Right epididymal diameter (cm)	1.42 ± 0.162^{a}	1.52 ± 0.080^{b}	1.24 ± 0.117^{c}	1.24 ± 0.117^{c}
Rel. right epididymal wgt (g)	0.142 ± 0.026^{a}	0.124 ± 0.025^{b}	0.134 ± 0.012^{c}	0.118 ± 0.016^{d}
Rel. left epididymal wgt (g)	0.160 ± 0.015^{a}	0.132 ± 0.018^{b}	0.130 ± 0.018^{c}	0.144 ± 0.025^{d}
Rel. total epididymal wgt (g)	0.302 ± 0.031^{a}	0.256 ± 0.043^{b}	0.264 ± 0.024^{c}	0.262 ± 0.039^{d}

Each result is mean ± SEM (n = 8). Results with similar superscripts $^{a\text{-}d}$ within rows are significantly different ($p < 0.05$). A: Olive oil (Control), B: Sodium arsenite (SA), C: crude extract of *Tridax procumbens* (CETP), and D: (B and C) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively.

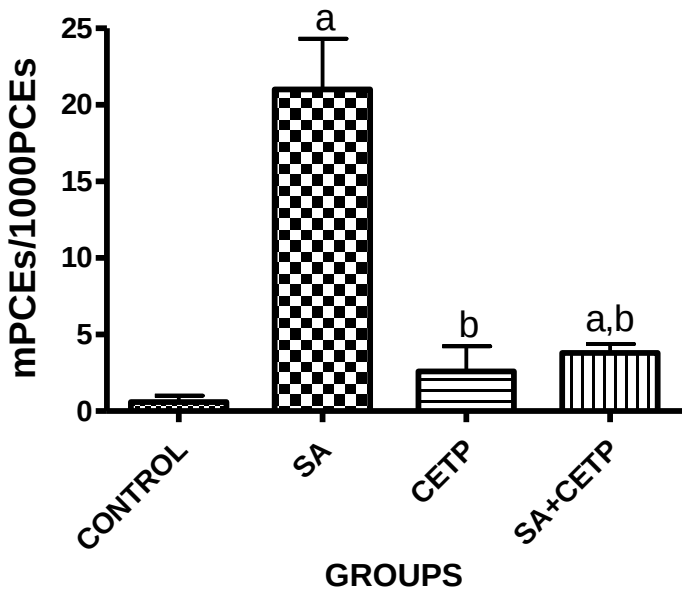


Figure 4.4. Comparative frequency of micronuclei formation in treated rats.

[a and b] significant ($p < 0.05$) from control and arsenite, respectively. Olive oil (Control), Sodium arsenite (SA) and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. n = 8.

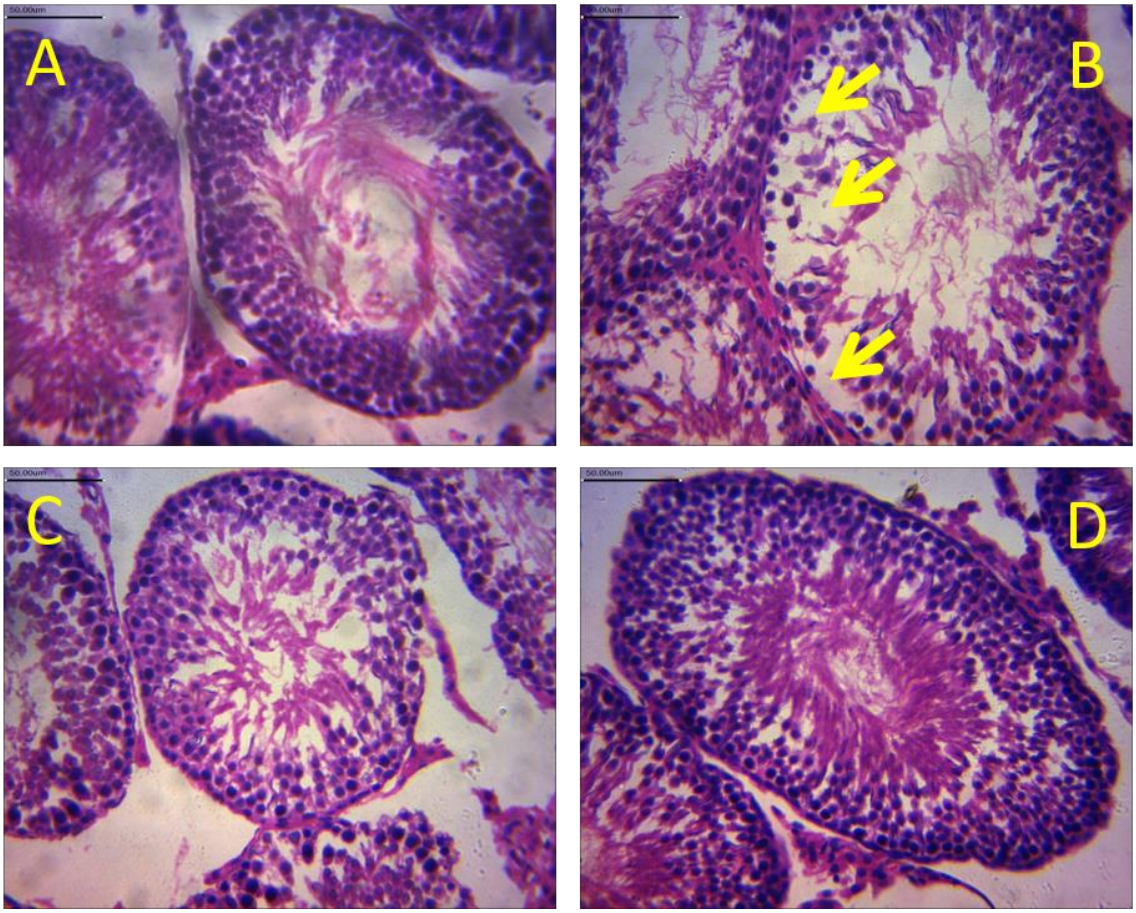


Plate 4.1. Representative photomicrographs of the testicular section of treated experimental rats. A (Control): No lesions found, B (SA): There is a moderate erosion of the germinal cells of the seminiferous tubules (arrow), C (CETP): No lesions found, D (CETP and SA): No lesions found. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 μm and indicate the magnification. n = 8.

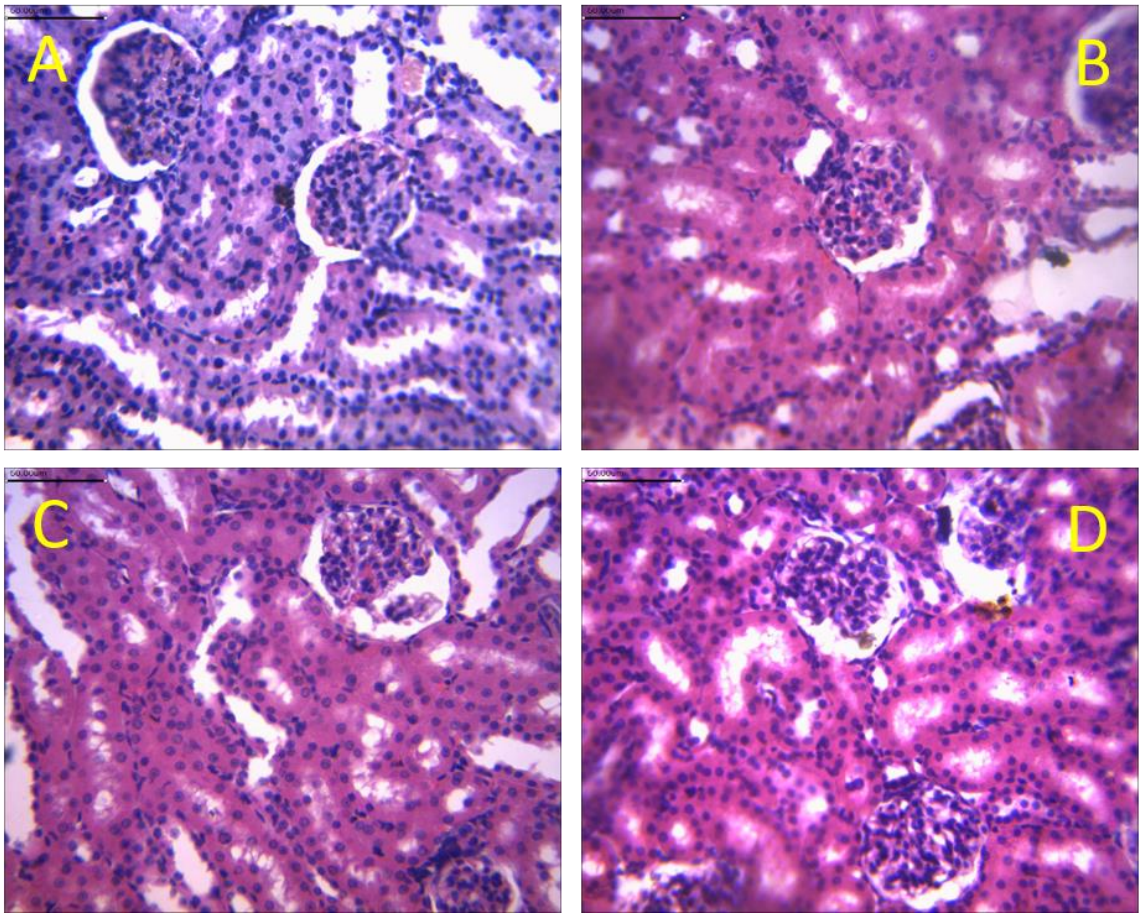


Plate 4.2. Representative photomicrographs of the kidney section of treated experimental rats. A (Control): No lesions found, B (SA): No lesions found, C (CETP): No lesions found, D (CETP and SA): No lesions found. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 µm and indicate the magnification. n =8.

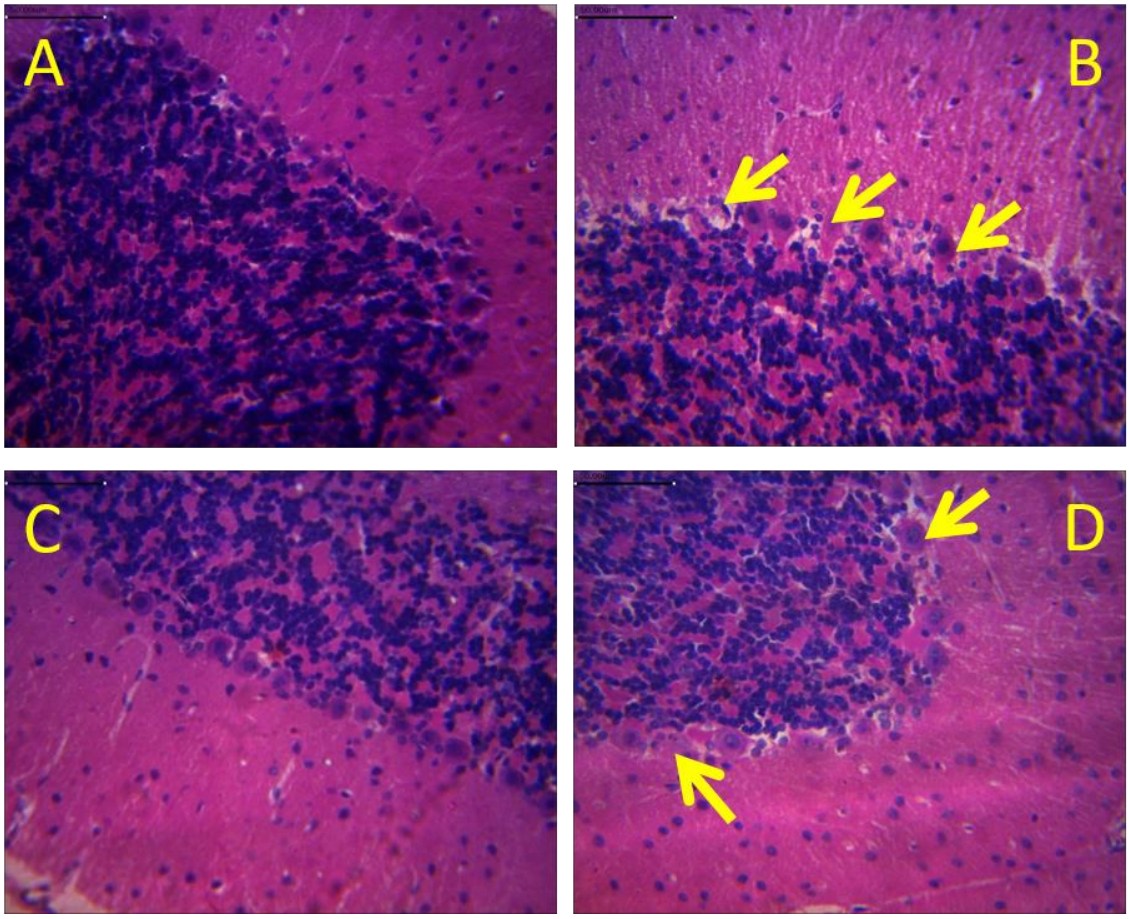


Plate 4.3. Representative photomicrographs of cerebellum section of treated experimental rats. A (Control): No lesions found, B (SA): The Purkinje cells are rounded (arrow) and degenerate (arrowhead), C (CETP): No lesions found, D (CETP and SA): Purkinje cells are rounded with reduced degeneration. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 µm and indicate the magnification. n =8.

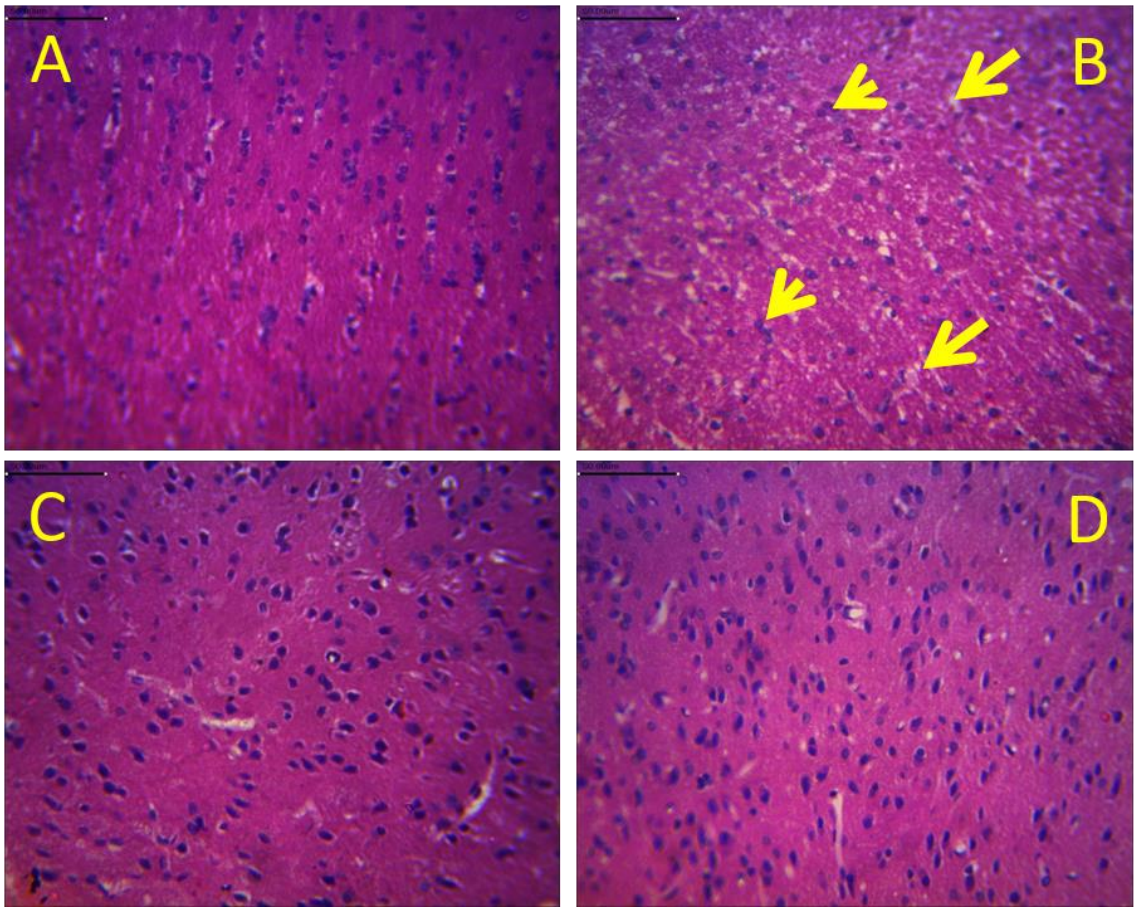


Plate 4.4. Representative photomicrographs of the cerebral cortex section of treated experimental rats. A (Control): No lesions found, B (SA): There is a diffuse spongiosis of the cerebral parenchyma (arrow) and gitter cell formation (arrowhead), C (CETP): No lesions found. D (CETP and SA): No visible lesions seen. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 µm and indicate the magnification. n =8.

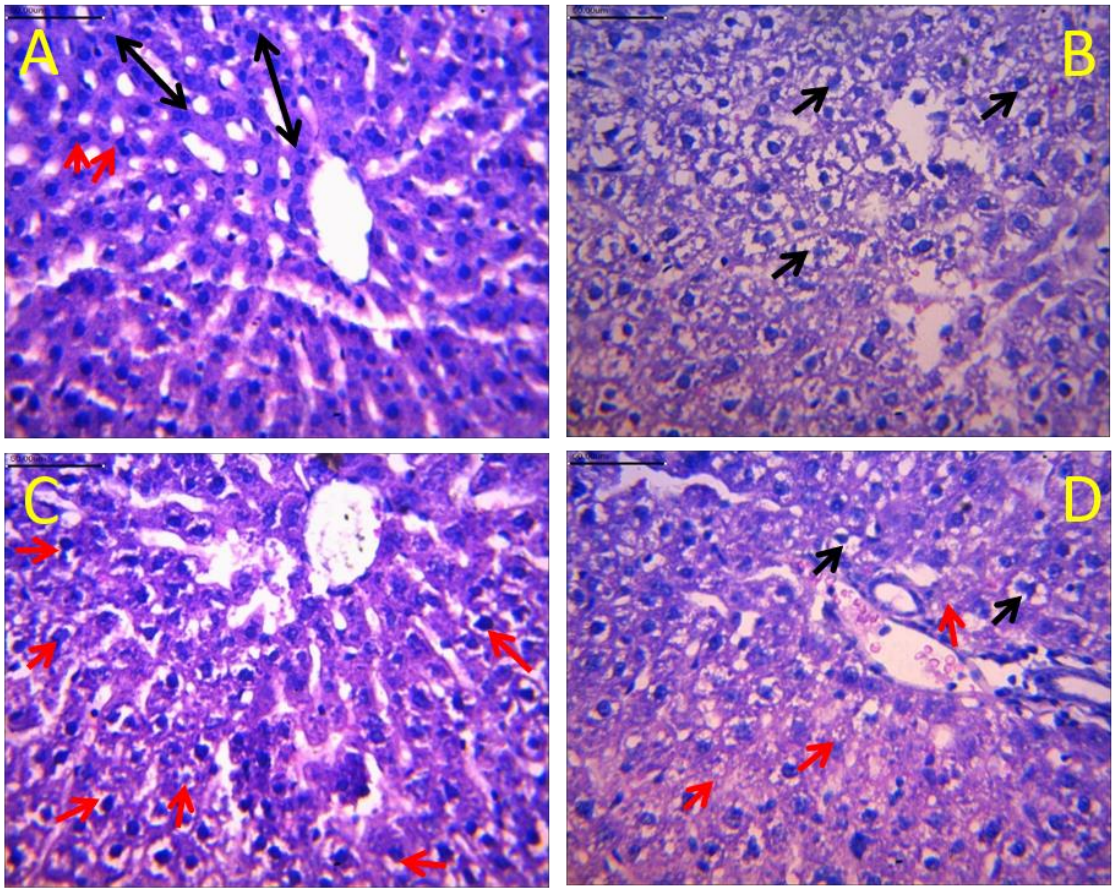


Plate 4.5. **Representative photomicrographs of the liver section of treated experimental rats.** A (Control): No lesion found, normal hepatocellular outline present, hepatic cords are visible (black double-pointed arrows) with scattered normal mitotic figures (red arrows), B (SA): Generalised severe hepatocellular degeneration evidenced by severe vacuolar/fatty degeneration, C (CETP): There is moderate mid-zonal hepatocellular vacuolar degeneration (red arrows), D (CETP and SA): Generalised moderate hepatocellular degeneration and sinusoidal congestion (red arrows) evidenced by vacuolar degeneration (black arrows), there is also severe disruption of hepatic cords. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 μm and indicate the magnification. n =8.

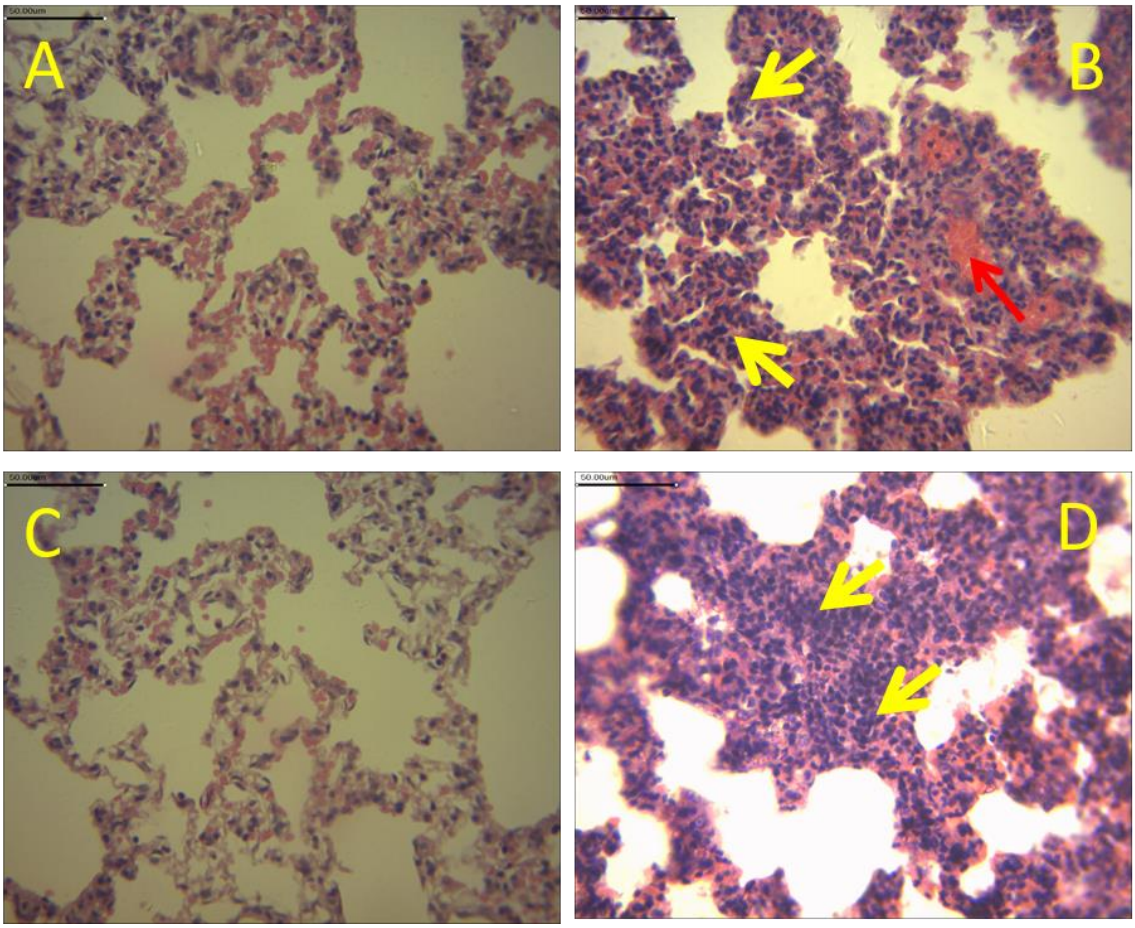


Plate 4.6. Representative photomicrographs of the lungs section of treated experimental rats. A (Control): No lesions found, B (SA): showing moderate interstitial congestion (yellow arrows) with the proliferation of alveolar pneumocytes and haemorrhage (red arrow), and thickened alveolar wall, C (CETP): No lesions found, D (CETP and SA There is a moderate congestion of the pulmonary interstitial with a thickened alveolar wall. Olive oil (Control), Sodium arsenite (SA), and crude extract of *Tridax procumbens* (CETP) were administered at 1 ml/kg (14 days), 0.0025 g/kg (7th and 14th day) and 0.05 g/kg (14 days), respectively. H&E stain. All scale bars are 50 μm and indicate the magnification. n =8.

4.3. Objective 3: Phytochemicals and antioxidant activities of *Tridax procumbens* leaf fractions

Flavonoids, terpenoids, tannins, steroids, alkaloids, saponins, anthraquinone, and phenols were seen following a qualitative phytochemical examination of *Tridax procumbens* extract and fractions (Table 4.11). However, quantitative analysis showed that the EA and DCM fractions possessed higher flavonoid and phenol contents compared to the crude extract and other fractions (Figures 4.5A and B).

Also, the antioxidant activities results showed that the EA and DCM fractions of *Tridax procumbens* possessed comparative scavenging activities on 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and nitric oxide (NO) radicals as the control (Figures 4.6A and B). The DCM scavenged DPPH and NO (IC_{50}: 0.43 and 0.39 μg/ml, respectively) relative to control (IC_{50}: 0.85 and 0.14 μg/ml, respectively) (Figures 4.6A and B). The EA and DCM fractions equally possessed higher total antioxidant capacity, and the ability to reduce ferric ion level compared to the crude extract (Figures 4.6C and D)

Table 4.11. Qualitative phytochemicals of *Tridax procumbens* extract and fractions

Phytochemicals	CETP	HXF	DCMF	EAF	AQF
Saponins	++	+	++	++	++
Tannins	+	-	+	++	++
Flavonoids	+	+	+	+	+
Anthraquinone	++	-	-	+	+
Cardiac glycoside	-	-	-	-	-
Terpenoids	+	+	+	+	+
Steroids	++	++	++	+	+
Alkaloids	+	+	+	+	+
Phenols	+	-	+	++	-

\+ = Present

++ = More abundant

\- = Absent

CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF and AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively. n = 3.

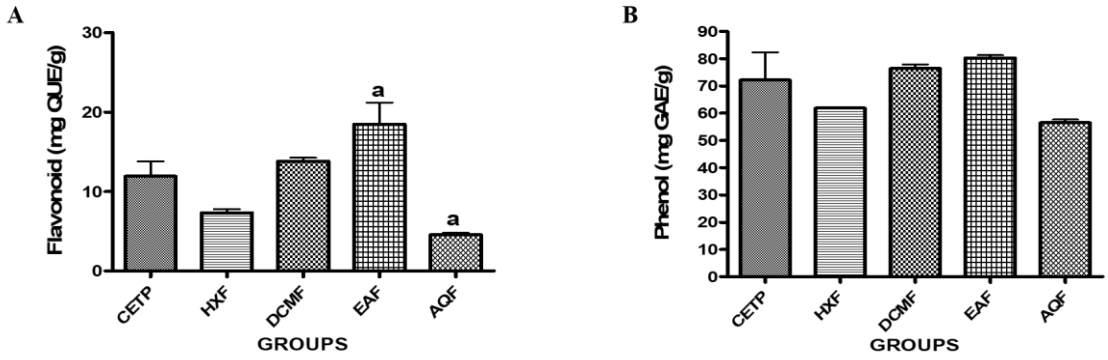


Figure 4.5. Quantitative phytochemicals of extract fractions of *Tridax procumbens*.

[a] Significant ($p < 0.05$) from CETP. CETP (crude extract of *Tridax procumbens*). HXF, DCMF, EAF and AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively. n = 3.

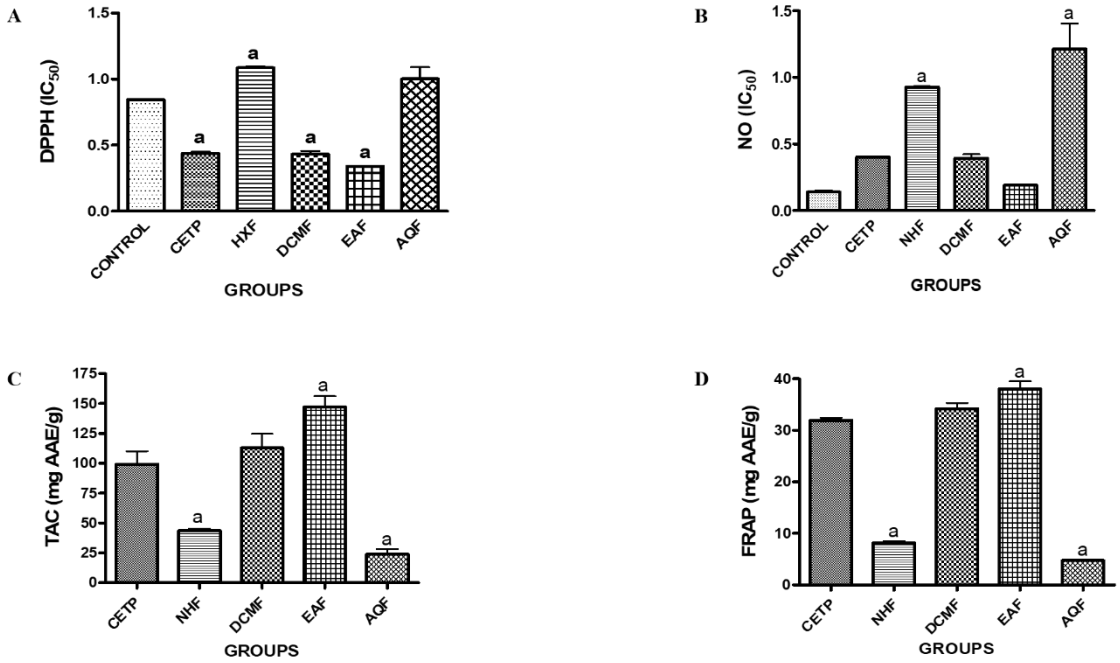


Figure 4.6. ***In vitro*** **antioxidant function of extract and fractions of** ***Tridax procumbens*****.**
For DPPH and NO: [a] Significant ($p < 0.05$) from control. For FRAP and TAC:
[a] Significant ($p < 0.05$) from CETP. CETP (crude extract of *Tridax procumbens*). HXF, DCMF, EAF and AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively. n = 3.

4.4. Objective 4: *Tridax procumbens* leaf extract and fractions elicited death in Panc-1 and HepG2 cells

Introduction

Cytotoxicity was evaluated using MTT, Trypan Blue, and Live-Dead assays on Panc-1 and HepG2 cell lines (Figures 4.7 - 4.12). Varying concentrations (10 - 250 μg/ml) of treatment with test samples after 24 and 48 hours were used.

The results showed that *Tridax procumbens* extract and fractions in a time and dose-dependent fashion attenuated both Panc-1 and HepG2 cell viability (Figures 4.7 - 4.12). Bioassay-directed extraction revealed that the dichloromethane fraction of *Tridax procumbens* exhibited the lowest IC_{50} value (23.1 μg/ml) relative to CETP (114.2 μg/ml), HXF (158.8 μg/ml), EAF (105.3 μg/ml) and AQF (251.1 μg/ml) using MTT test (Figure 4.8B).

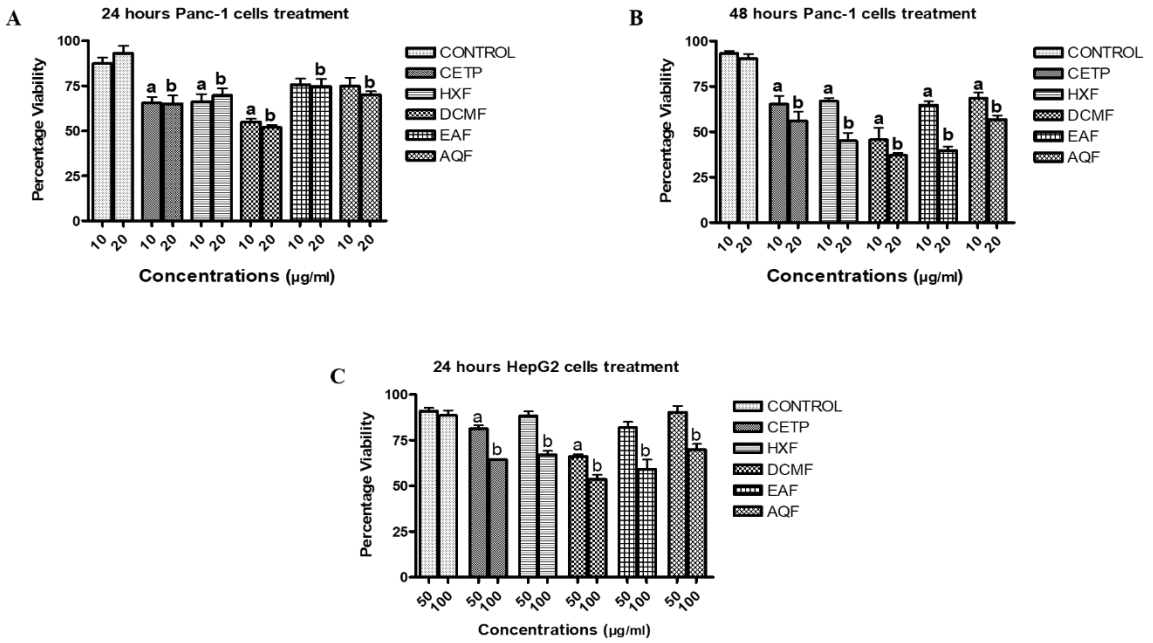


Figure 4.7. *Tridax procumbens* extract and fractions elicited Panc-1 and HepG2 cells death using Trypan blue assay after 24 and 48-hour treatment. The dichloromethane fraction shows greater reduced cell viability. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.

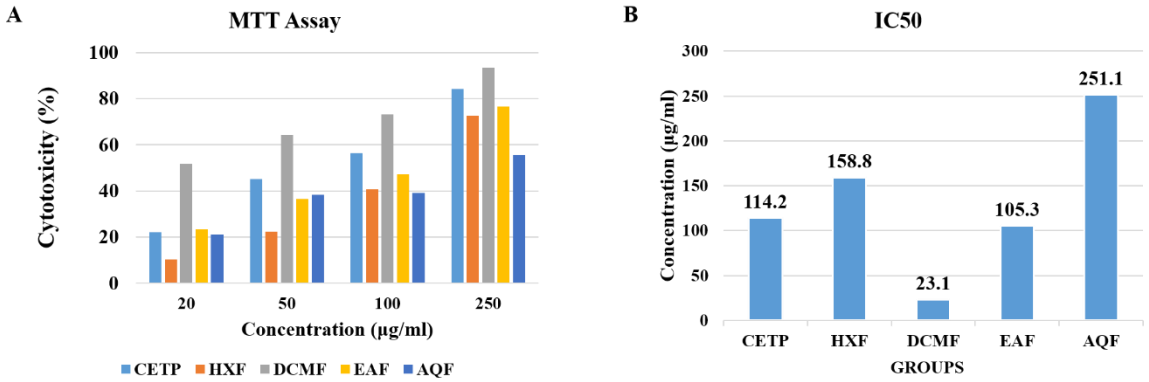


Figure 4.8. *Tridax procumbens* extract and fractions elicited Panc-1 cell death using MTT assay after 24-hour treatment. The dichloromethane fraction is more cytotoxic with the lowest IC_{50} value. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.

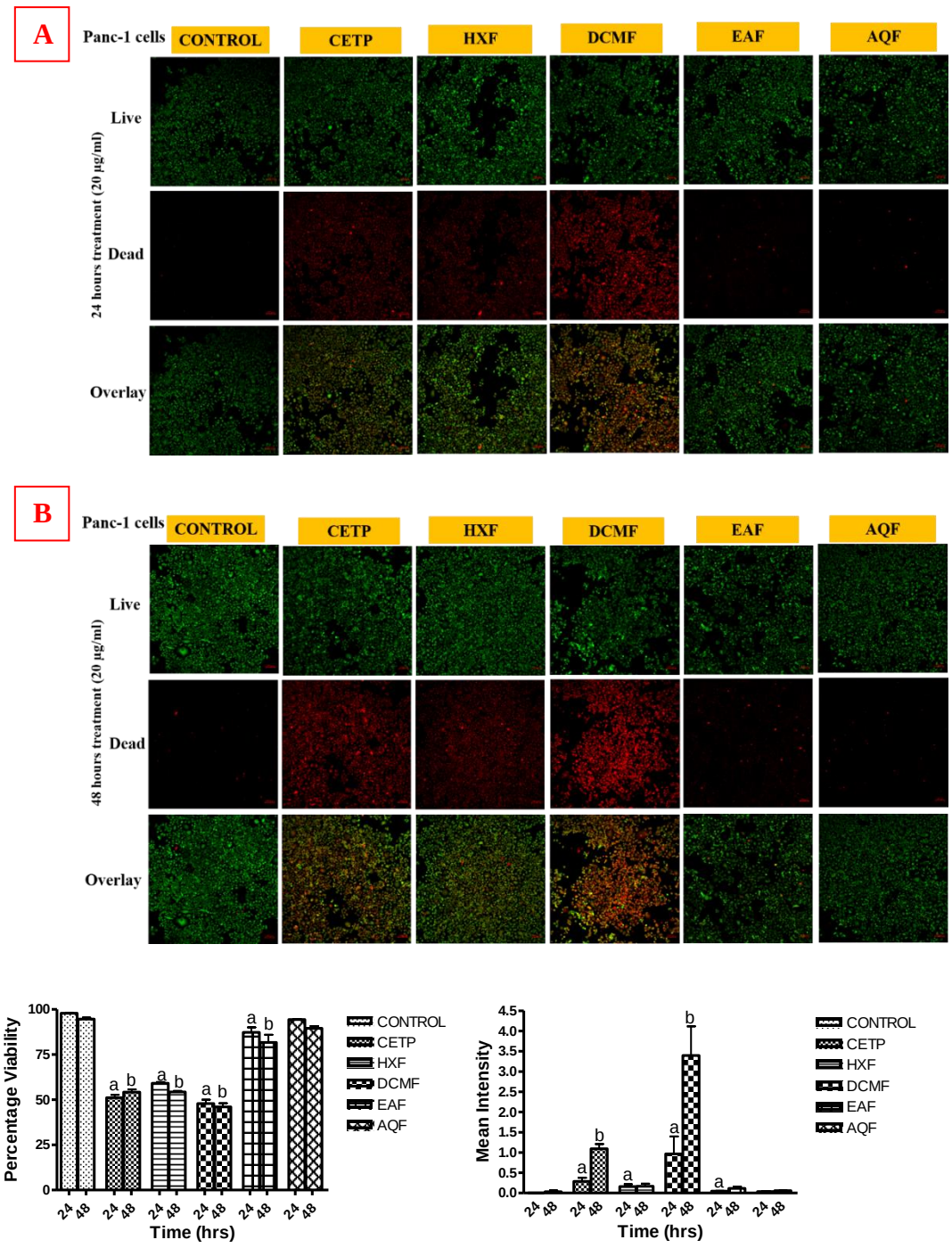


Figure 4.9. ***Tridax procumbens*** **extract and fractions elicited Panc-1 cell death using calcein AM and ethidium homodimer-1 cytotoxicity kit.** [a] significant ($p < 0.05$) from control after 24-hour treatment (20 μg/ml). [b] significant ($p < 0.05$) from control after 48-hour treatment (20 μg/ml). Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 100 µm and indicate the magnification. n = 3.

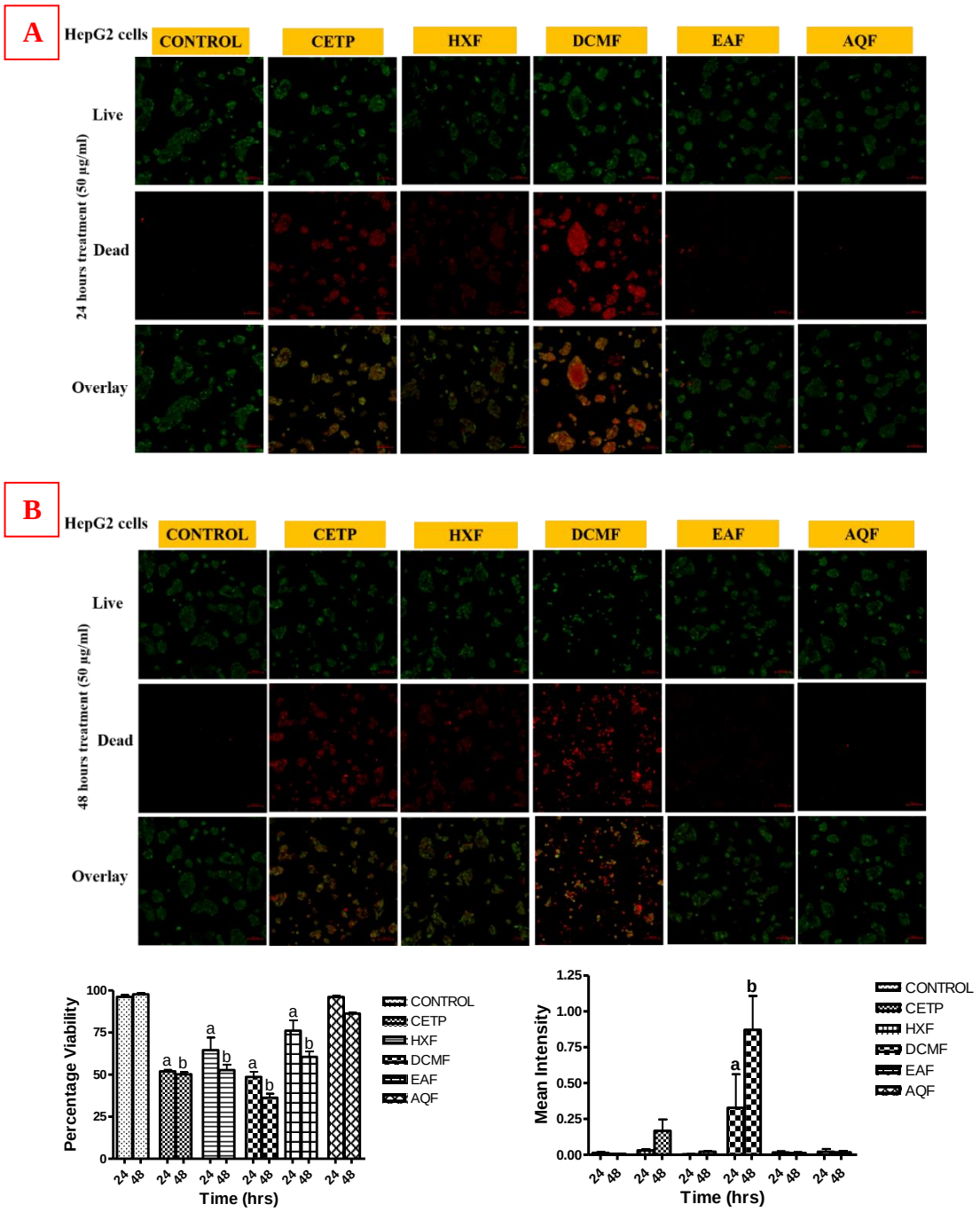


Figure 4.10. *Tridax procumbens* extract and fractions (50 μg/ml) elicited HepG2 cell death using calcein AM and ethidium homodimer-1 cytotoxicity kit. [a] significant from control after 24-hour treatment (50 μg/ml). [b] significant from control after 48-hour treatment (50 μg/ml). Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 100 µm and indicate the magnification. n = 3.

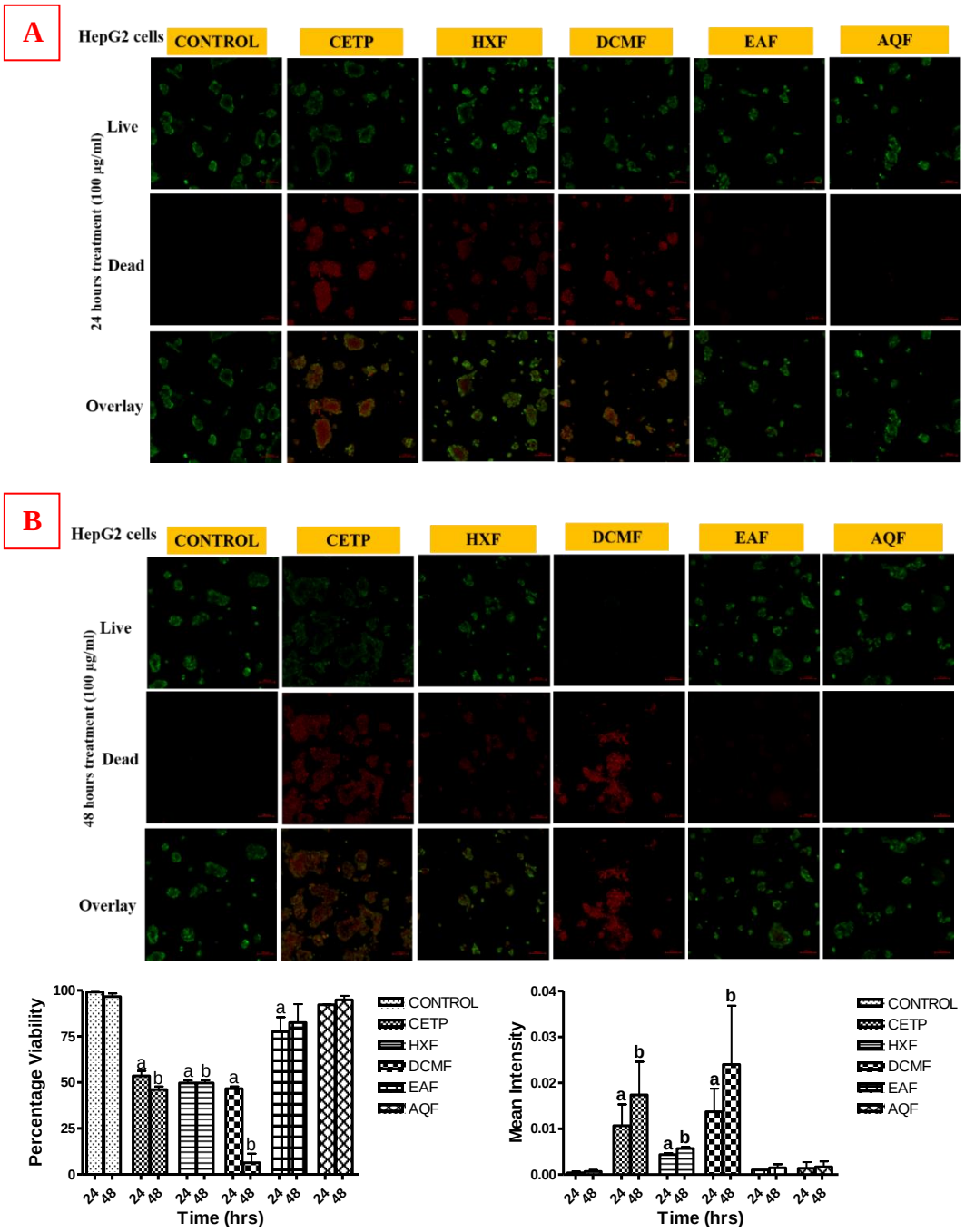


Figure 4.11. *Tridax procumbens* extract and fractions (100 µg/ml) elicited HepG2 cell death using calcein AM and ethidium homodimer-1 cytotoxicity kit. [a] significant ($p < 0.05$) from control after 24-hour treatment (100 µg/ml). [b] significant ($p < 0.05$) from control after 48-hour treatment (100 µg/ml). Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 100 µm and indicate the magnification. n = 3.

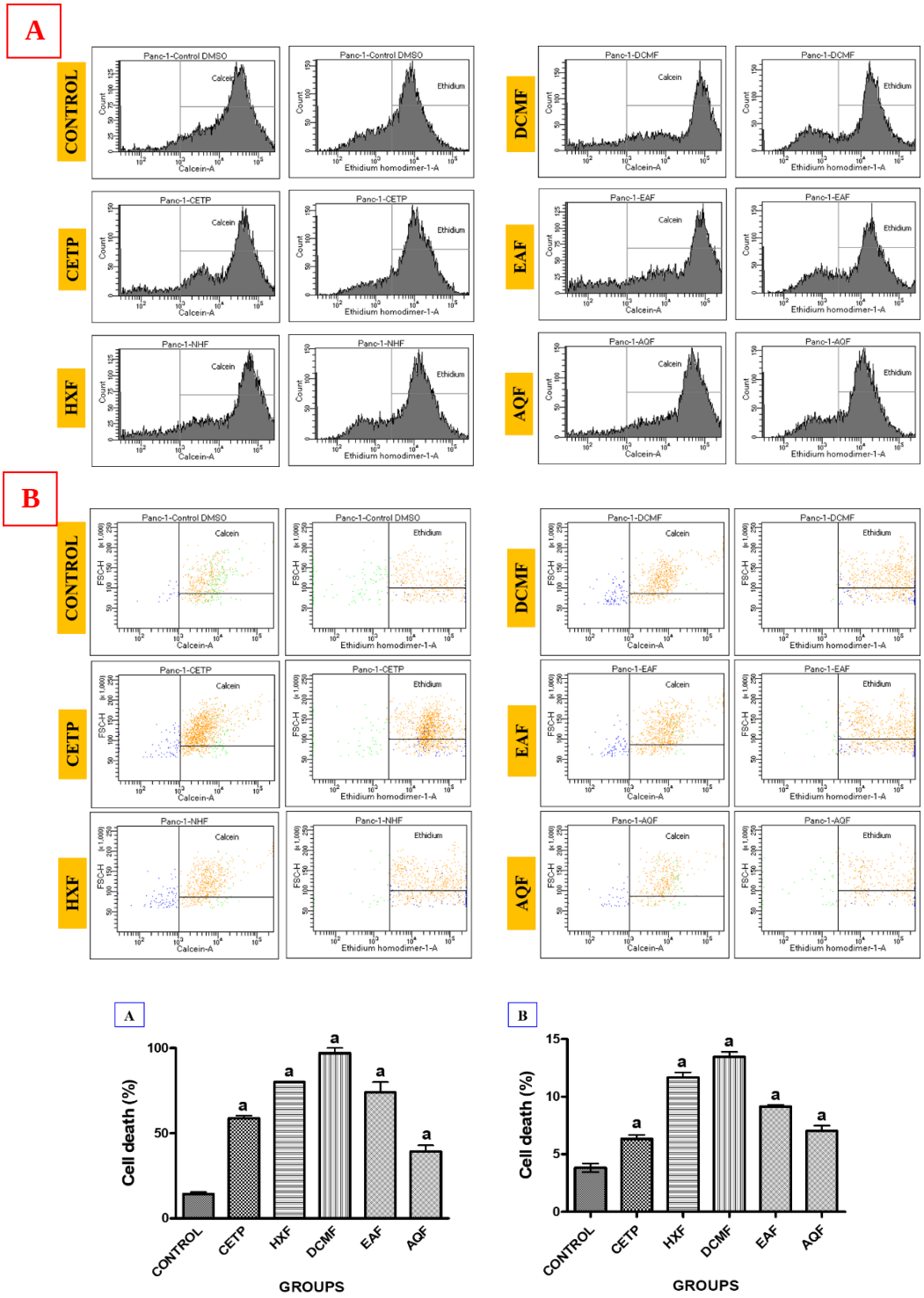


Figure 4.12. ***Tridax procumbens*** **extract and fractions elicited Panc-1 cell death using flow cytometry method.** [a] significant ($p < 0.05$) from control after 24-hour treatment (20 μg/ml). Bar graph A (both dying and dead cells with compromised membrane), Bar graph B (dead cells with compromised membrane). Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.

4.5. Objective 5: *Tridax procumbens* leaf fraction caused phenotype morphogenesis, modulates peristalsis and upregulates markers specific to embryonic mouse pancreas and liver

Figure 4.13 - 4.16 shows the various organs that were obtained (E11.5 day) and cultured for 5 days. The embryonic mouse tissues were treated with extract and fractions of *Tridax procumbens* for 48 hours, fixed, and immunostained for proteins specific to the tissue type. Markers of the various pancreatic cells, insulin, glucagon, and somatostatin (endocrine cell); amylase (exocrine cell); cytokeratin 7 and peanut agglutinin (ductal cell); and vimentin (mesenchymal cell) (Figures 4.18 - 4.22) coupled with alpha-fetoprotein (Figure 4.17) and E-cadherin (Figure 4.24) for the liver and intestine, respectively were monitored.

There was increased expression of alpha-fetoprotein in the crude extract, dichloromethane, and ethyl acetate fractions-treated liver tissue (Figure 4.17A). At higher magnification, the test samples, especially the dichloromethane fraction, produced a phenotype of the hepatocytes forming strands (Figure 4.17B). In addition, there was immunopositivity for insulin, glucagon, somatostatin, amylase, cytokeratin 7, peanut agglutinin, and vimentin in the extract and fractions treated groups (Figures 4.18 - 4.22). However, it was observed that there was enhanced branching morphogenesis of the ductal system in the DCMF-treated cultured embryonic pancreas based on increased expression of cytokeratin 7 and peanut agglutinin (Figures 4.20 and 4.21). There was a paradoxical effect of the dichloromethane fraction on the percentage contraction of cultured embryonic mouse intestine and oesophagus (E11.5d) after treatment (20 μg/ml) for 48 hours (Figure 4.23).

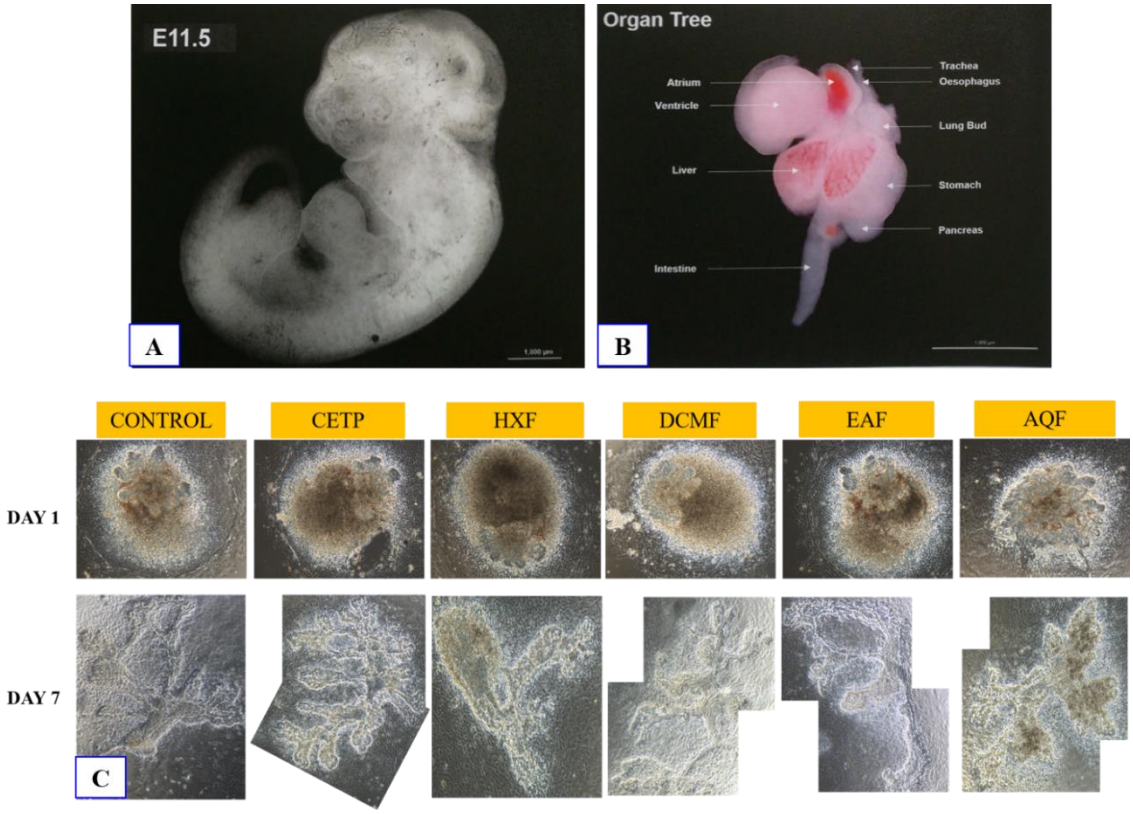


Figure 4.13. Photomicrograph of embryonic mouse pancreas (E11.5d) cultured for 5 days and treated (20 µg/ml) for 48 hours. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.

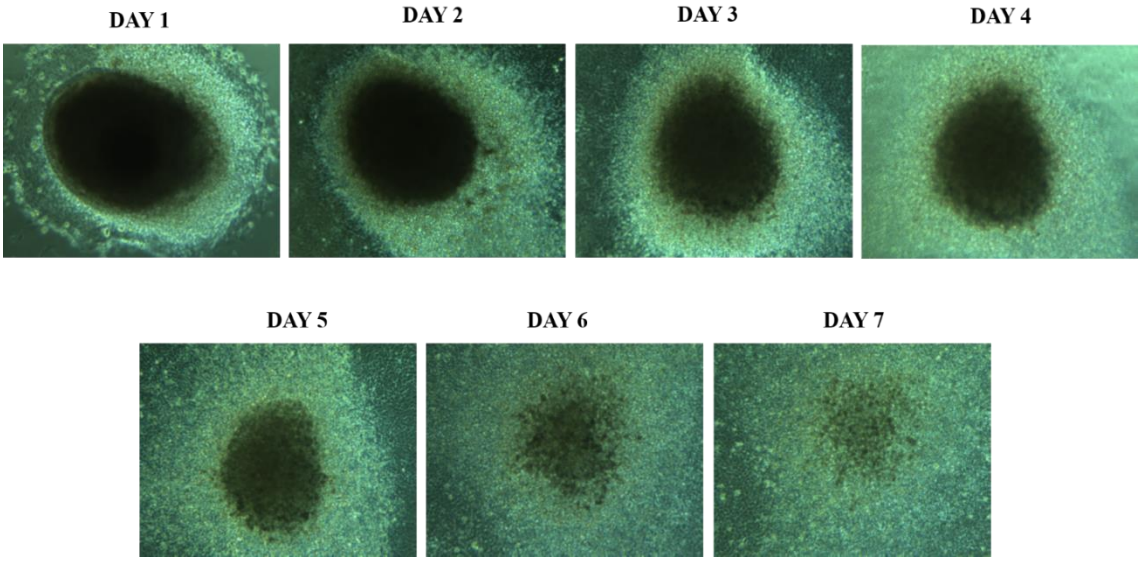


Figure 4.14. Photomicrograph of cultured embryonic mouse liver (E11.5d) from day 1 to day 7.

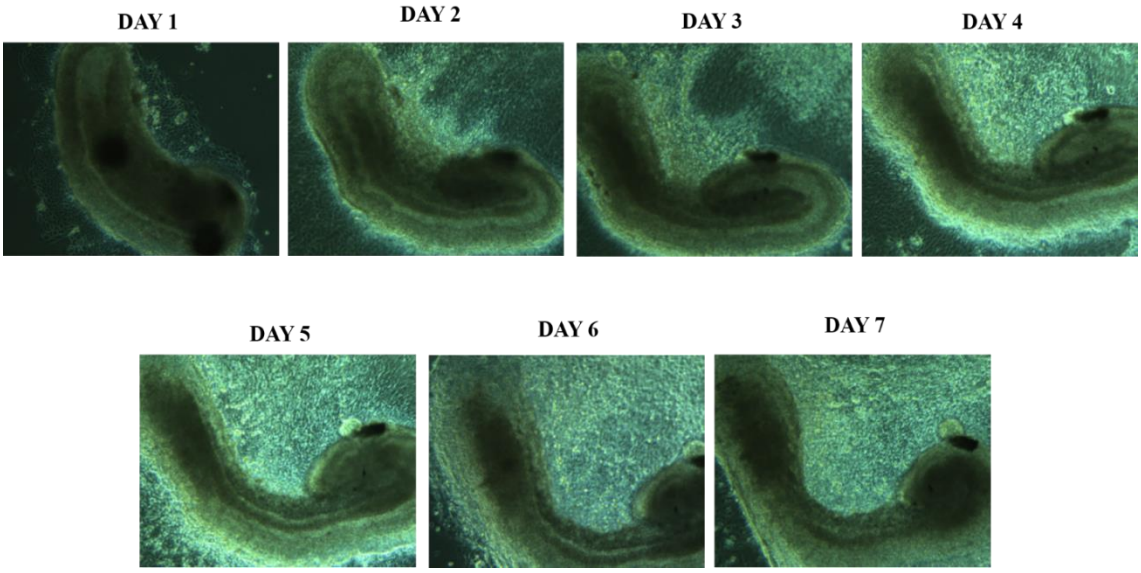


Figure 4.15. Photomicrograph of cultured embryonic mouse intestine (E11.5d), day 1 to day 7.

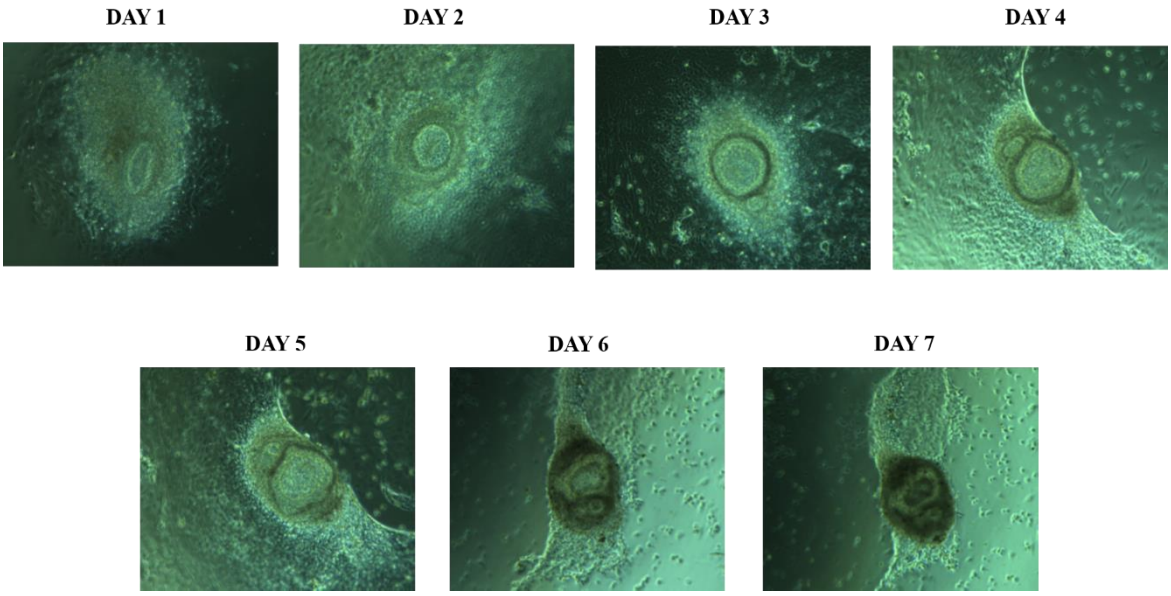


Figure 4.16. Photomicrograph of cultured embryonic mouse oesophagus (E11.5d), day 1 to day 7.

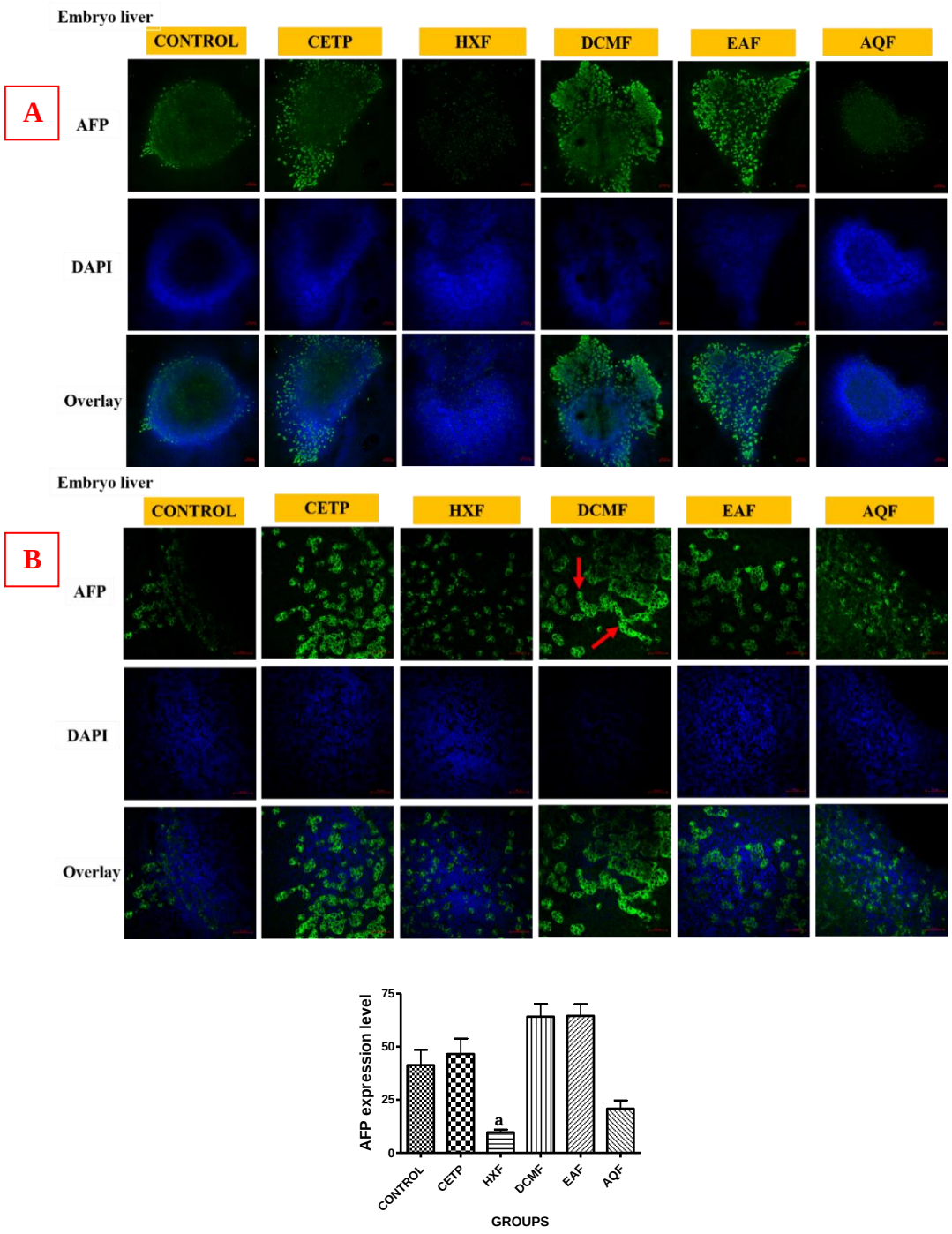


Figure 4.17. The extract and fractions of *Tridax procumbens* stained positive for alpha fetoprotein in embryonic mouse liver (A and B) after treatment (20 µg/ml) for 48 hours. CETP and DCMF showed more AFP expression; however, DCMF produced a phenotype of the hepatocytes forming strands in the embryonic mouse liver (arrow in B). Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars indicate the magnification (100 µm (A), 50 µm (B)). n = 3.

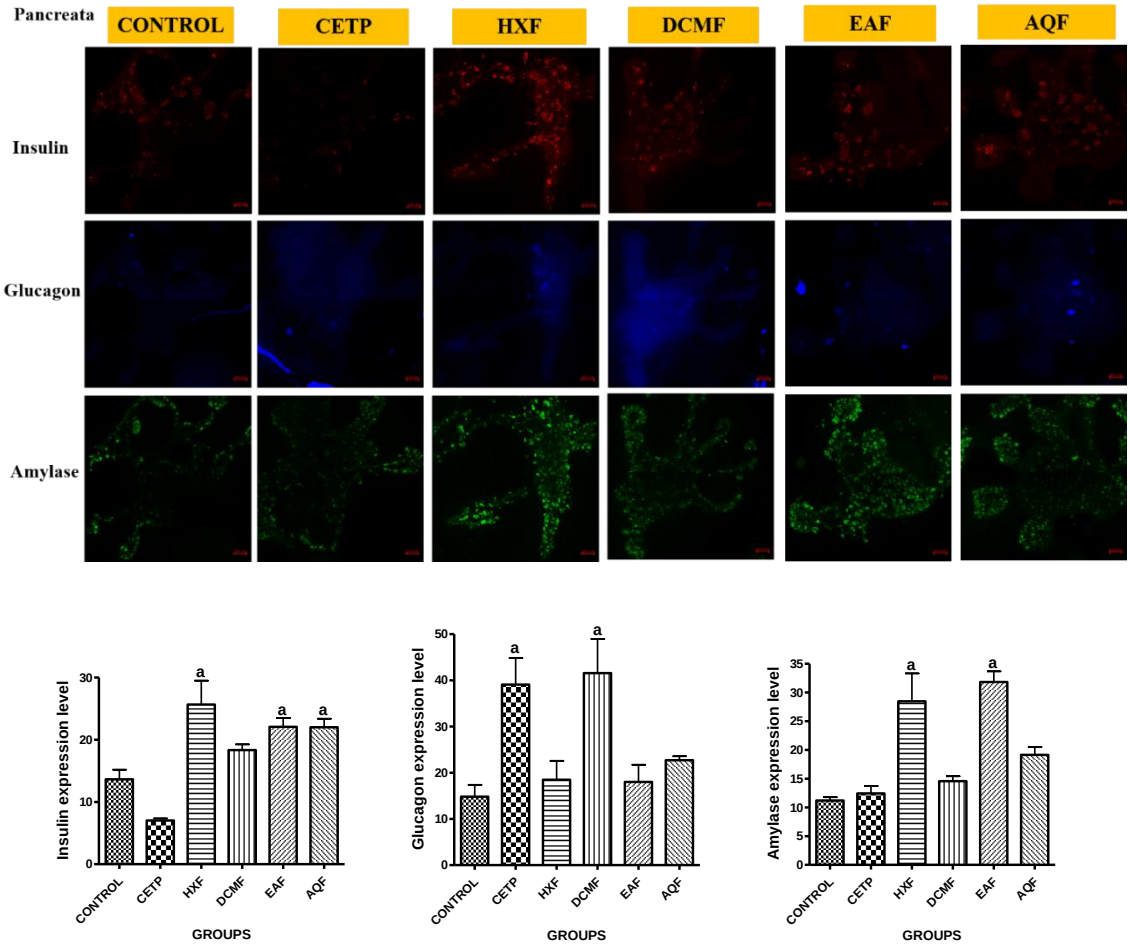


Figure 4.18. The extract and fractions of *Tridax procumbens* stained positive for insulin, glucagon and amylase in embryonic mouse pancreas after treatment (20 µg/ml) for 48 hours. HXF and DCMF treated groups revealed even positivity for the three stains in the embryonic mouse pancreas. However, HXF treated group showed more positivity for insulin and amylase compared to the control and other test groups. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 100 µm and indicate the magnification. n = 3.

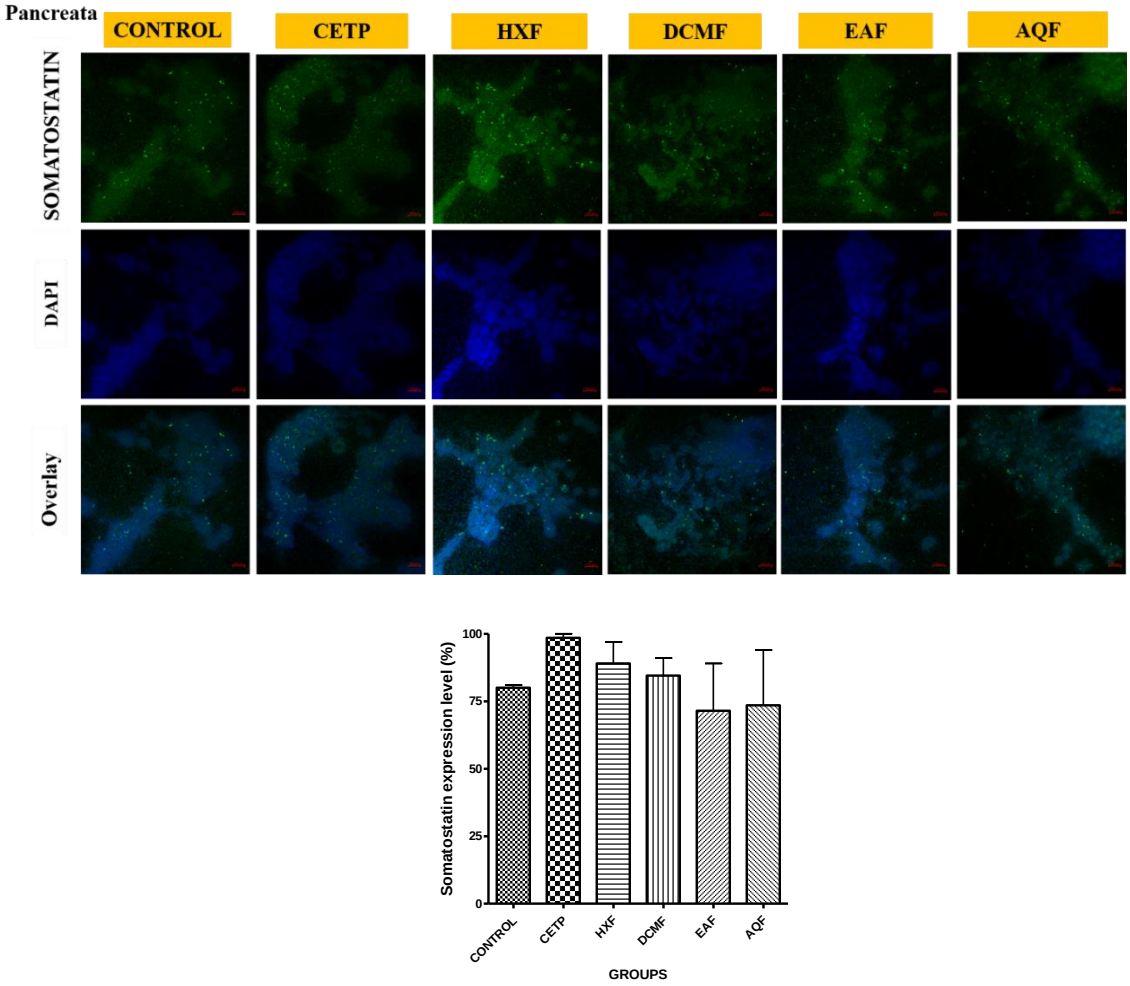


Figure 4.19. The extract and fractions of *Tridax procumbens* stained positive for somatostatin in embryonic mouse pancreas after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 100 µm and indicate the magnification. n = 3.

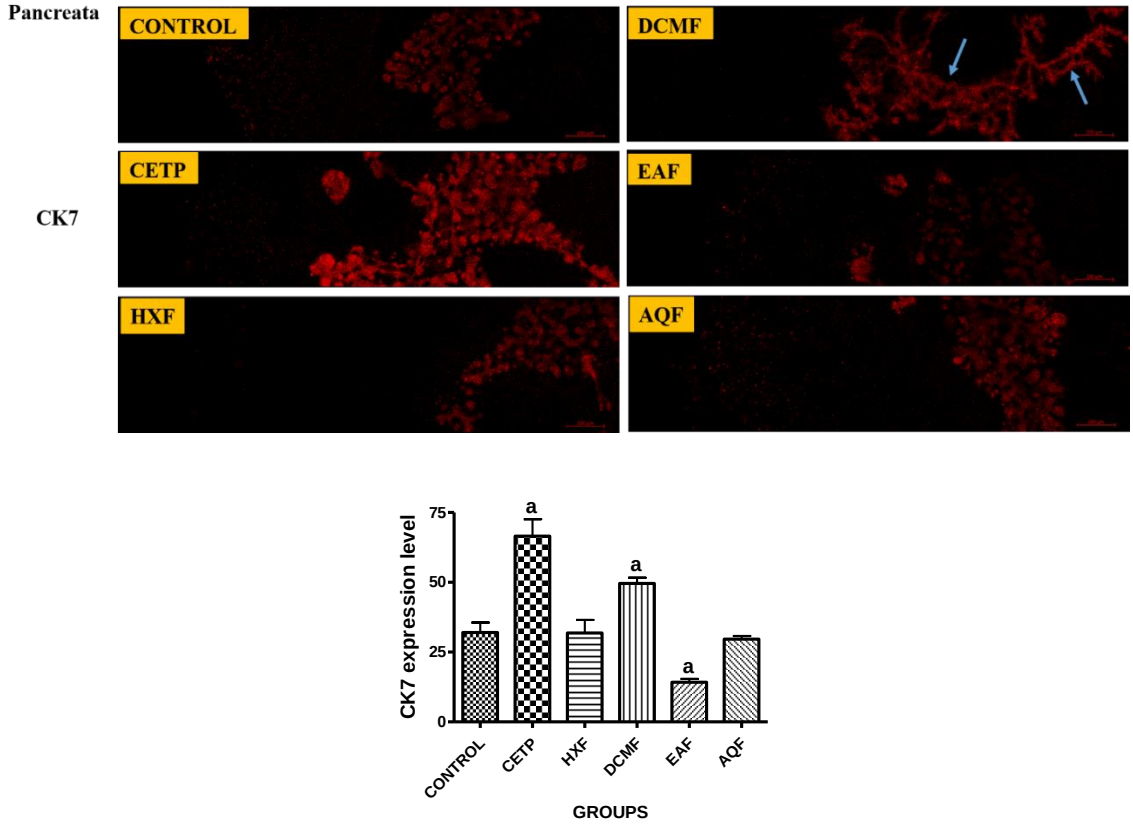


Figure 4.20. The extract and fractions of *Tridax procumbens* stained positive for cytokeratin 7 (CK7) in embryonic mouse pancreas (arrow) after treatment (20 µg/ml) for 48 hours. CETP and DCMF show more positivity; however, DCMF produced a more branching morphogenesis phenotype in the embryonic mouse pancreas. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 200 µm and indicate the magnification. n = 3.

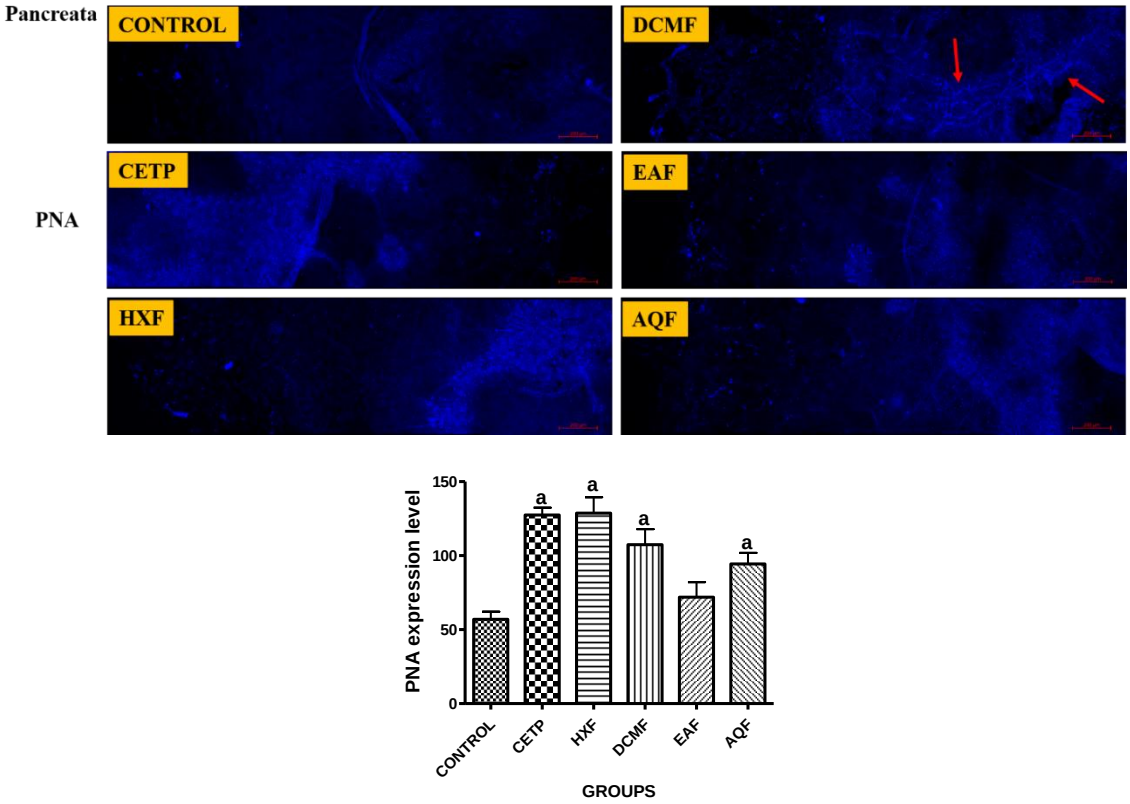


Figure 4.21. The extract and fractions of *Tridax procumbens* stained positive for peanut agglutinin (PNA) in embryonic mouse pancreas (arrow) after treatment (20 µg/ml) for 48 hours. CETP and DCMF show more positivity; however, DCMF produced a more branching morphogenesis phenotype in the embryonic mouse pancreas. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 200 µm and indicate the magnification. n = 3.

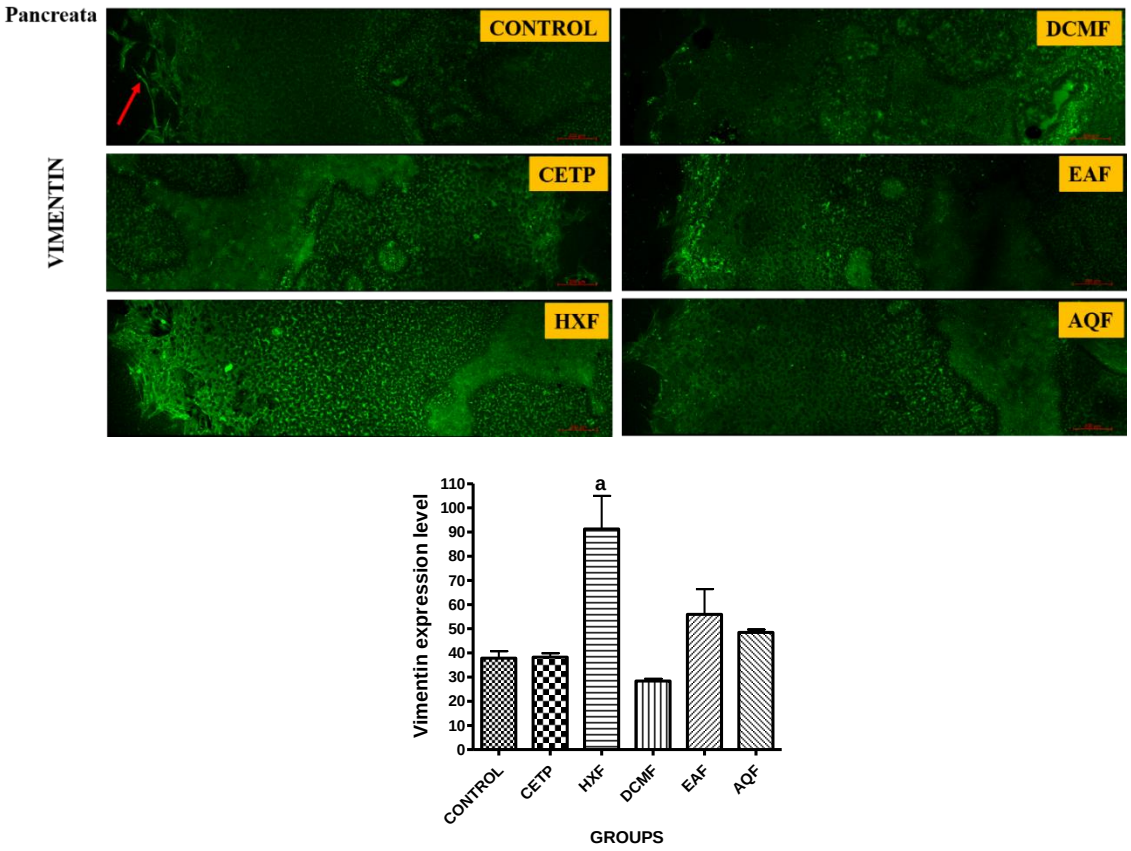


Figure 4.22. The extract and fractions of *Tridax procumbens* stained positive for vimentin in embryonic mouse pancreas (arrow) after treatment (20 µg/ml) for 48 hours. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). Scale bars are 200 µm and indicate the magnification. n = 3.

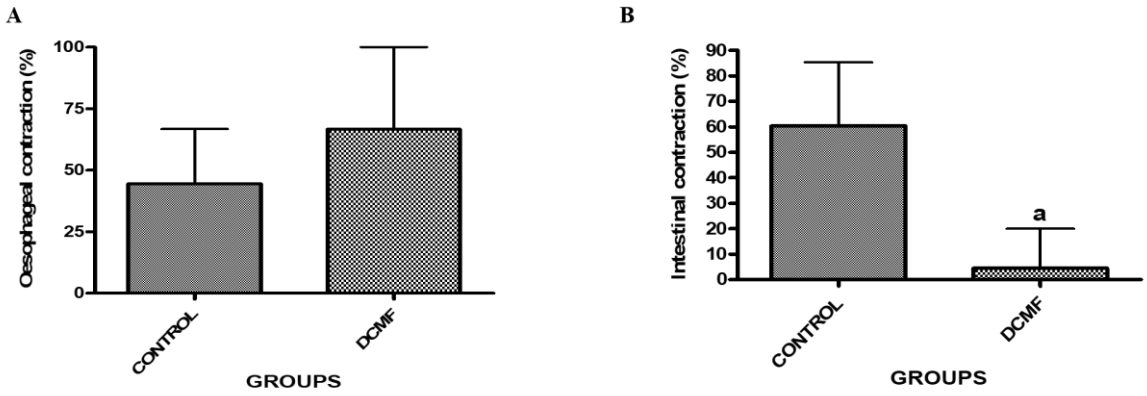


Figure 4.23. Paradoxical effect of dichloromethane fraction of *Tridax procumbens* on percentage contraction of cultured embryonic mouse intestine and oesophagus (E11.5d) after treatment (20 µg/ml) for 48 hours. Control: dimethyl sulfoxide, DCMF: dichloromethane fraction of *Tridax procumbens*. n = 3.

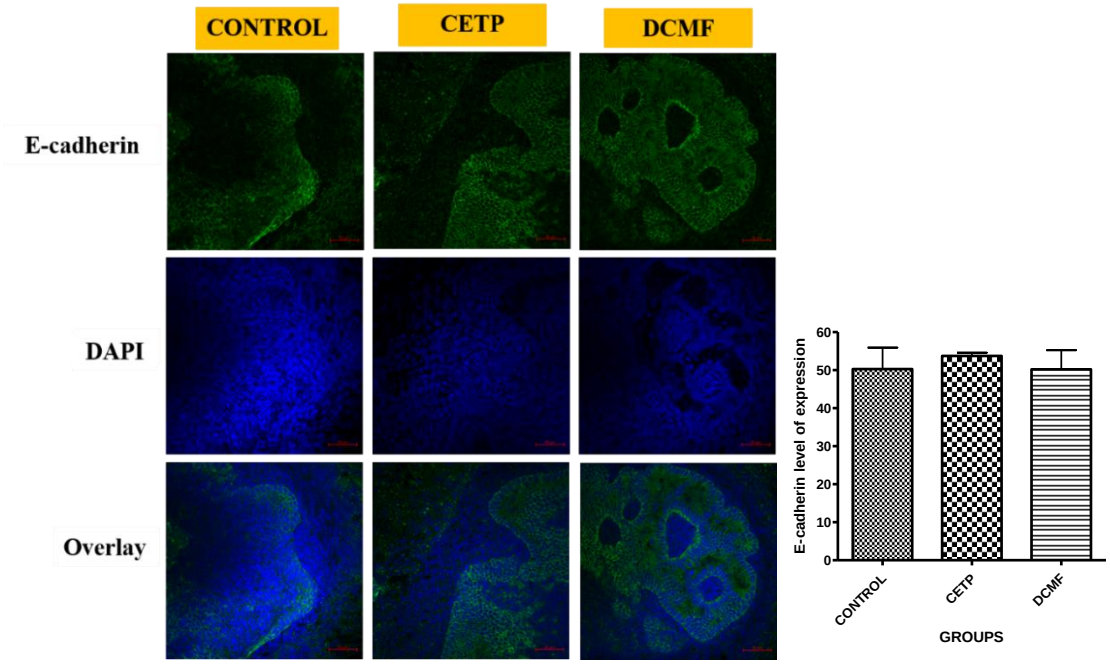


Figure 4.24. The crude extract and dichloromethane fraction of *Tridax procumbens* stained positive for E-cadherin in the embryonic mouse intestine after treatment (20 µg/ml) for 48 hours. Control: dimethyl sulfoxide, CETP: crude extract of *Tridax procumbens*, DCMF: dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

4.6: Objective 6: *Tridax procumbens* fraction suppresses Panc-1 and HepG2 cells via markers of oxidative stress, apoptosis, proliferation, differentiation and metastasis

The rich phytocompounds and anti-oxidative stress activities of *Tridax procumbens* dichloromethane fraction in addition to its cytotoxicity to Panc-1 cells warranted the suggestion that it could also possess the potential to induce endogenous compounds or antioxidant enzymes. However, it was discovered in this present experiment that the Panc-1 cell treated with dichloromethane fraction displayed a significant reduction in GST-pi activities (Figure 4.29) and elevation in the antioxidant function of both alkaline phosphatase (Figure 4.30) and catalase enzymes (Figure 4.31).

Meanwhile, it was hypothesised that the cytotoxic potential of the dichloromethane fraction is due to apoptosis, hence, Bcl-2 and caspase-3 expression were monitored (Figures 4.27 and 4.28). The DCMF induced the expression level of caspase-3 (Figure 4.27), and Bcl-2 (Figure 4.28).

However, it was observed that there was cytoplasmic localisation of the Bcl-2 with the control, and both cytoplasmic and punctate perinuclear localisation for the dichloromethane fraction (Figure 4.28). After the Panc-1 and HepG2 cells were exposed to the extract and fractions of *Tridax procumbens*, there was significant upregulation of APC, p53, and p21 protein in the DCMF-exposed Panc-1 cells comparable to control (Figures 4.32, 4.25, and 4.26).

However, DCMF increased significantly the set of cells positive for $p21^{Cip1/Wap-1}$ (Figure 4.26). Downregulation of nuclear localisation and enhanced cytoplasmic staining of adenomatous polyposis coli when the Panc-1 cells were treated with dichloromethane fraction (Figure 4.32) was observed.

Conversely, there was significant downregulation of CK7, PNA, vimentin, Ki-67, and CAII proteins in the DCMF-treated Panc-1 cells (Figures 4.36, 4.37, 4.35, 4.38, and 4.39), and β-catenin, Sox9, AAT, AFP (Figures 4.33, 4.34, 4.42, and 4.43) in the HepG2 cells when compared with control. Meanwhile, DCMF produced more cytoplasmic expression of β-catenin relative to the nuclear localisation in the control, and also reduced the nuclear expression level of Sox9 protein to a more cytoplasmic

staining pattern (Figures 4.33 and 4.34). The level of albumin and transferrin expression in the DCMF-treated HepG2 cells increased significantly (Figures 4.40 and 4.41).

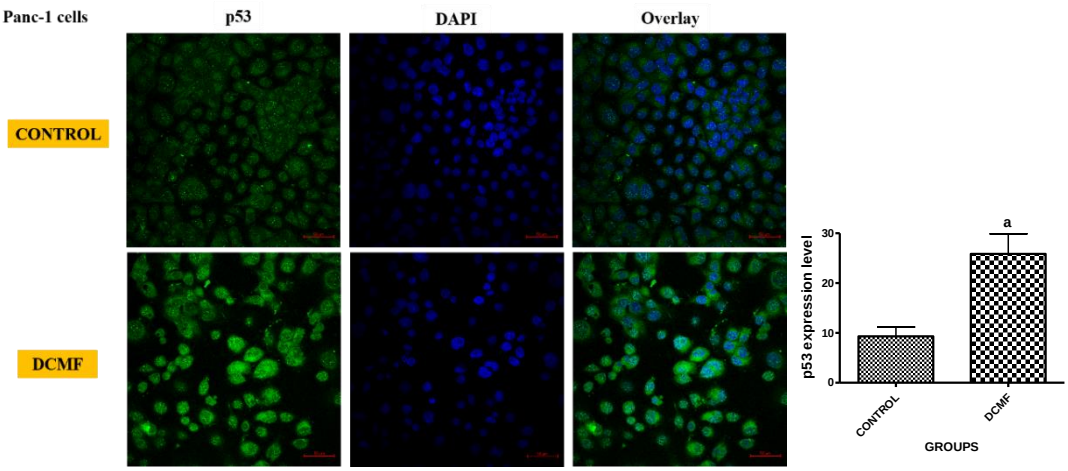


Figure 4.25. The dichloromethane fraction of *Tridax procumbens* upregulates p53 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

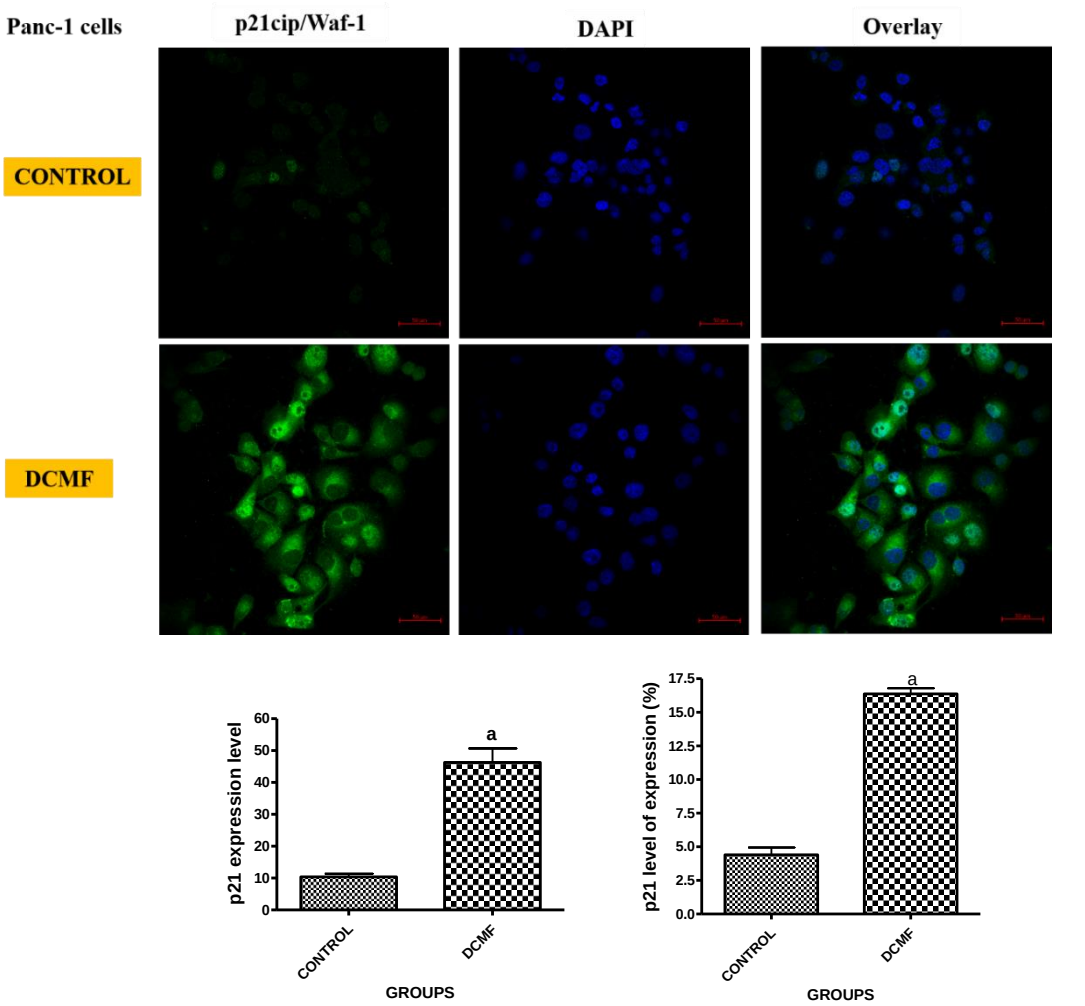


Figure 4.26. The dichloromethane fraction of *Tridax procumbens* upregulates p21 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

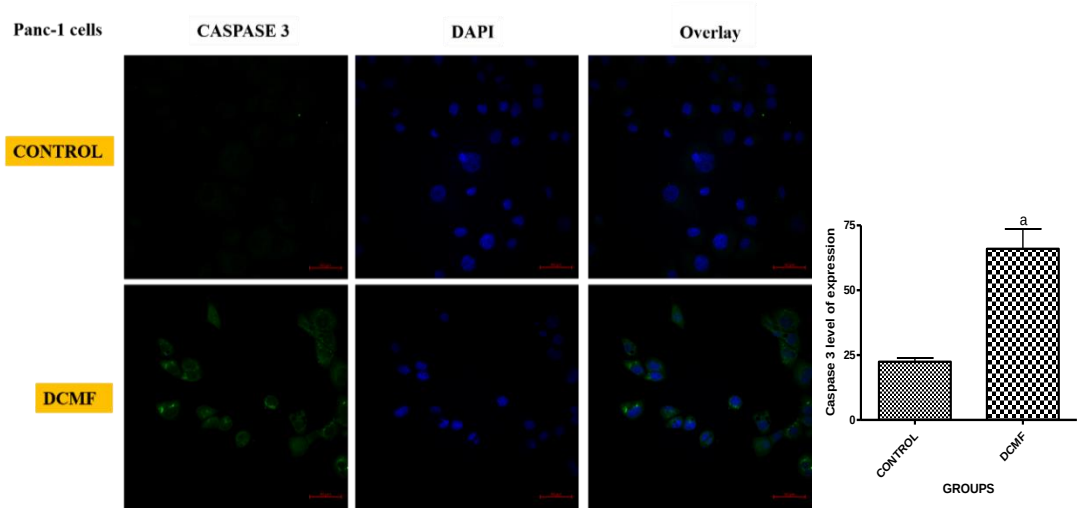


Figure 4.27. The dichloromethane fraction of *Tridax procumbens* upregulates caspase3 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

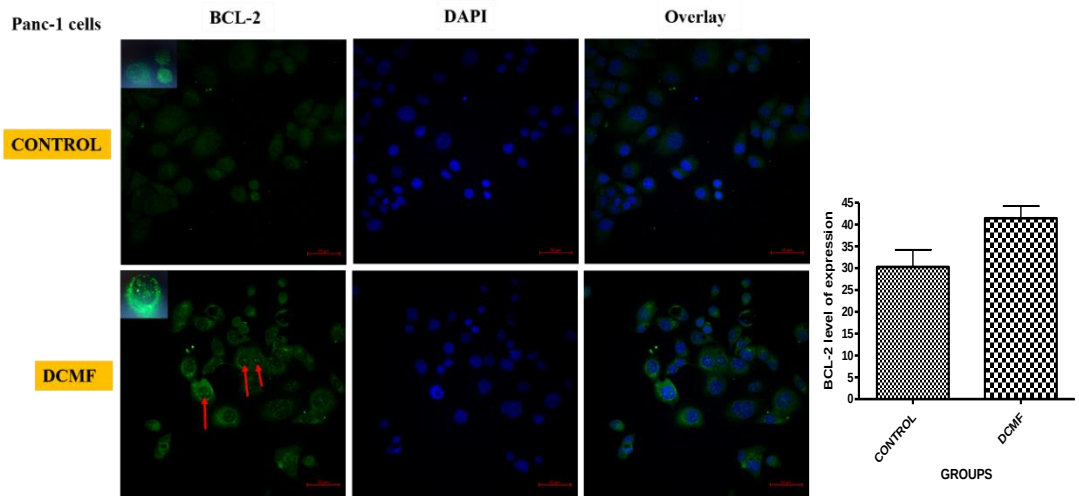


Figure 4.28. The dichloromethane fraction of *Tridax procumbens* produced a perinuclear and punctate nuclear (red arrows, insert) pattern of staining for Bcl-2 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different (p < 0.05) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

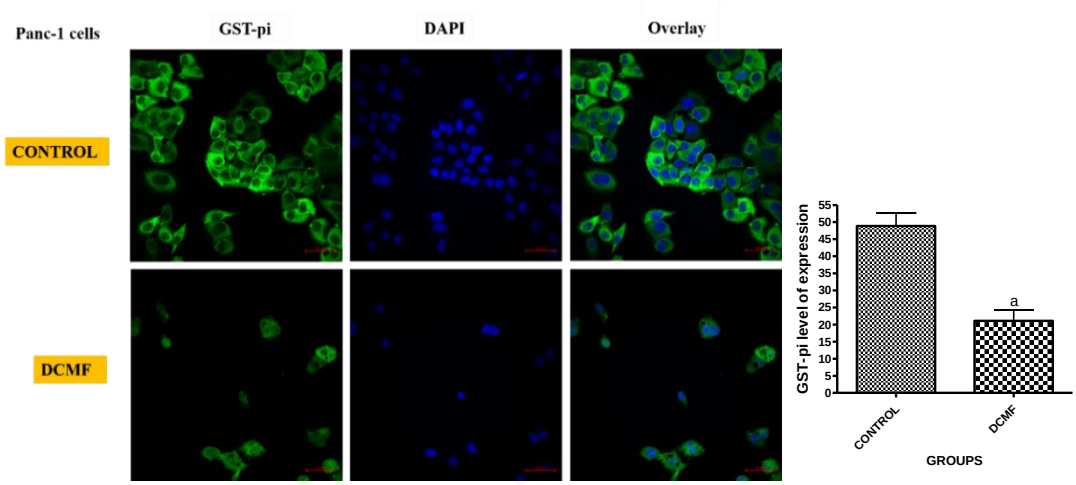


Figure 4.29. The dichloromethane fraction of *Tridax procumbens* reduced GST-pi activity in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

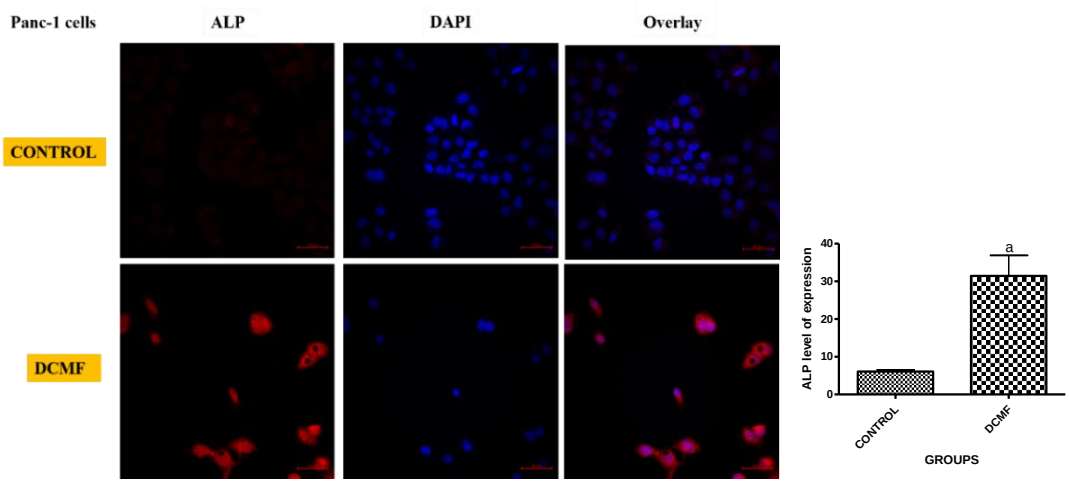


Figure 4.30. The dichloromethane fraction of *Tridax procumbens* increased alkaline phosphatase (ALP) activity in Panc-1 cells after treatment (20 µg/ml) for 48 hours.

[a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

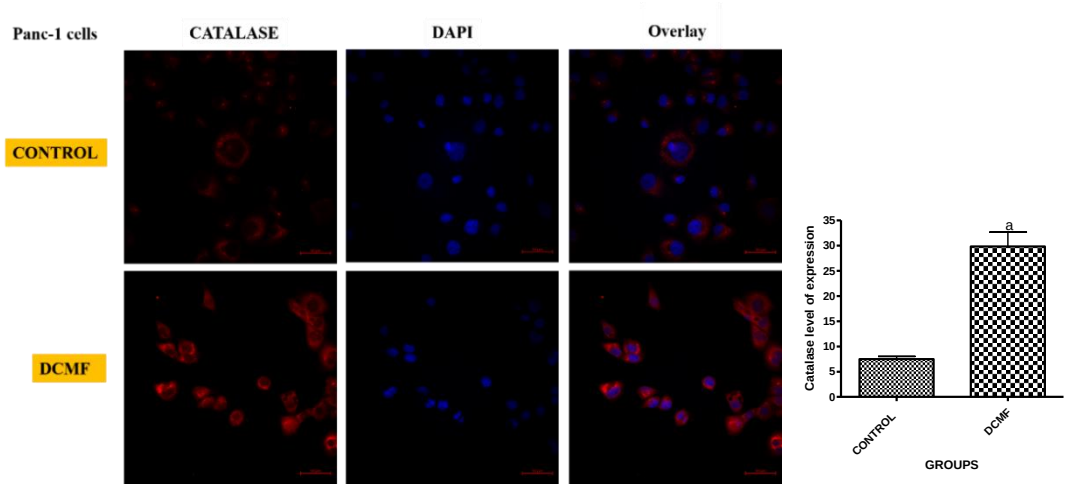


Figure 4.31. The dichloromethane fraction of *Tridax procumbens* increased catalase activity in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

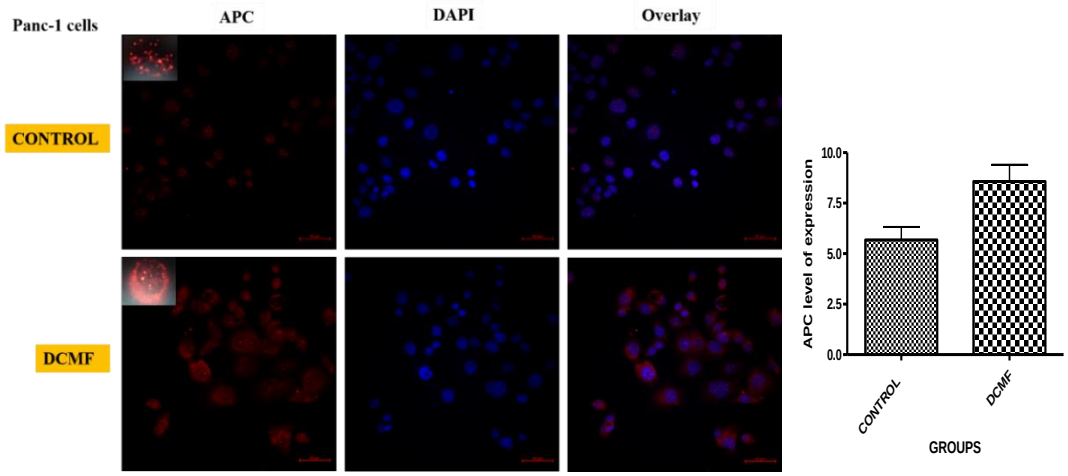


Figure 4.32. The dichloromethane fraction of *Tridax procumbens* upregulates adenomatous polyposis coli (APC) in Panc-1 cells after treatment (20 µg/ml) for 48 hours. The DCM-treated cells showed cytoplasmic expression for APC while the control cells showed nuclear APC staining (insert). [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

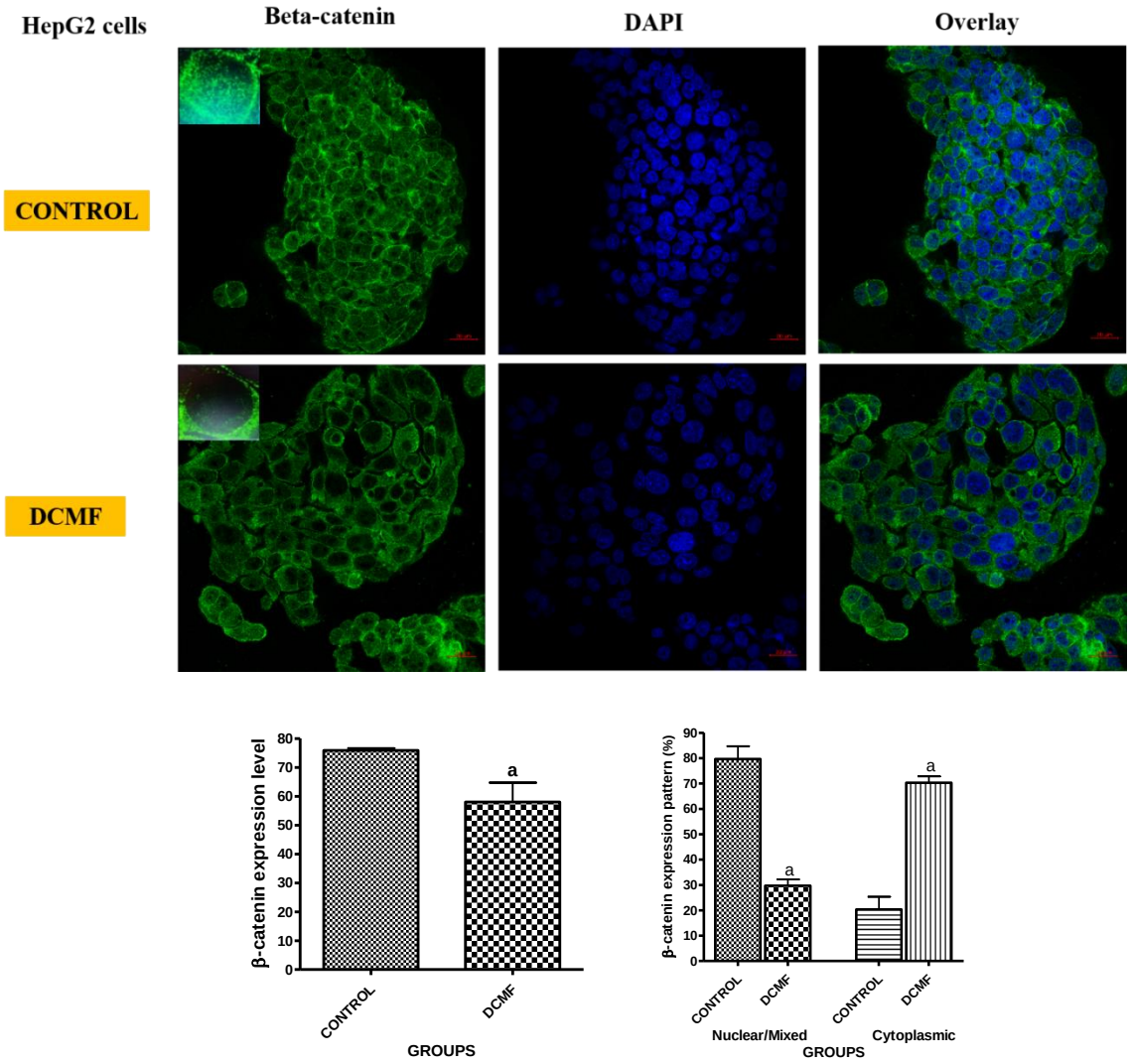


Figure 4.33. The dichloromethane fraction of *Tridax procumbens* downregulates beta-catenin in HepG2 cells after treatment (20 µg/ml) for 48 hours. There was also enhancement of cytoplasmic staining of the protein in the DCMF-treated group (insert). [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 20 µm and indicate the magnification. n = 3.

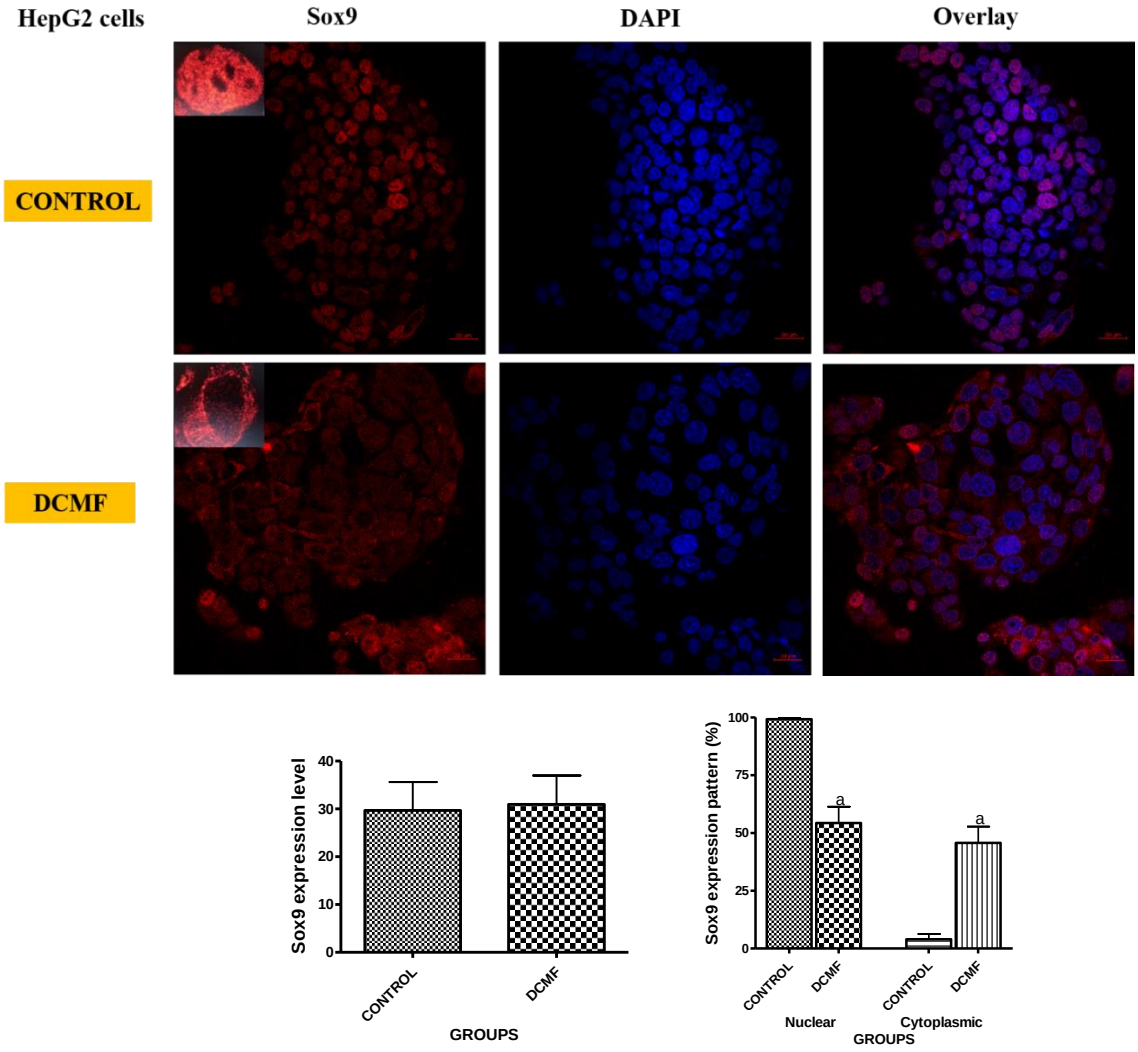


Figure 4.34. The dichloromethane fraction of *Tridax procumbens* downregulates Sox9 in HepG2 cells after treatment (20 µg/ml) for 48 hours. There was significant translocation of Sox9 to the cytoplasm in the DCMF-treated group (insert). [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 20 µm and indicate the magnification. n = 3.

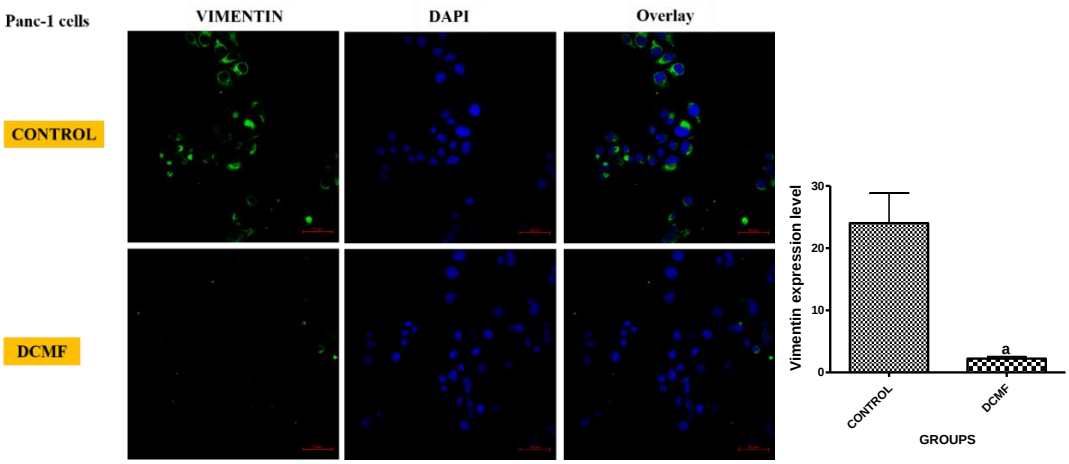


Figure 4.35. The dichloromethane fraction of *Tridax procumbens* downregulates vimentin in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

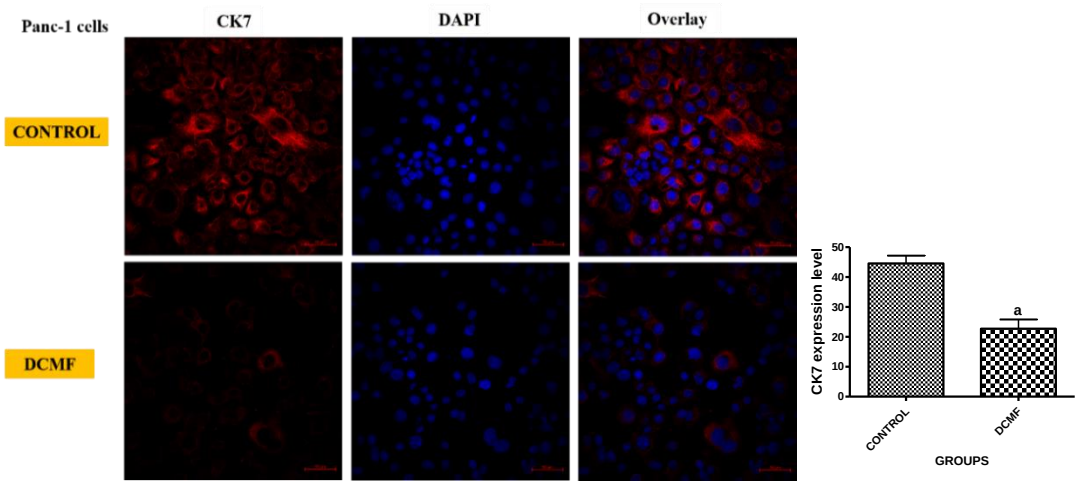


Figure 4.36. The dichloromethane fraction of *Tridax procumbens* downregulates cytokeratin 7 (CK7) in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

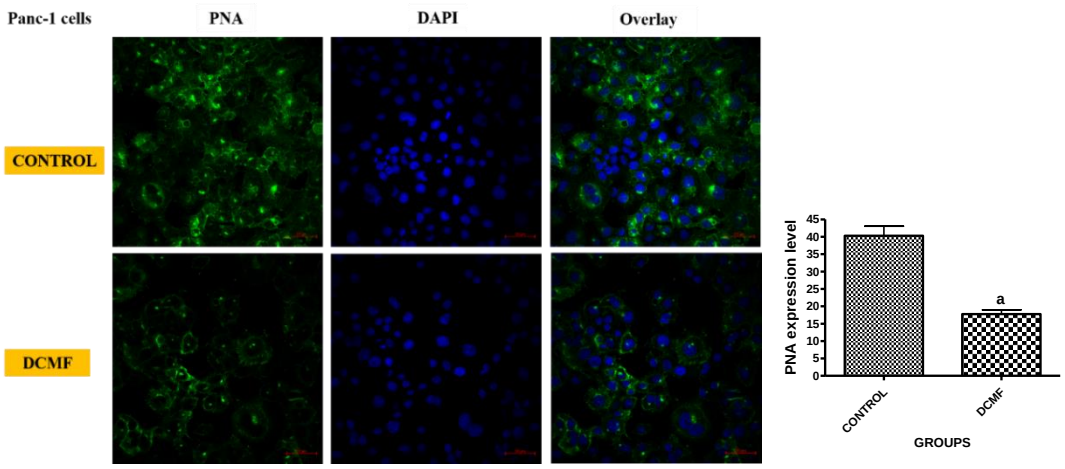


Figure 4.37. The dichloromethane fraction of *Tridax procumbens* downregulates peanut agglutinin (PNA) in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

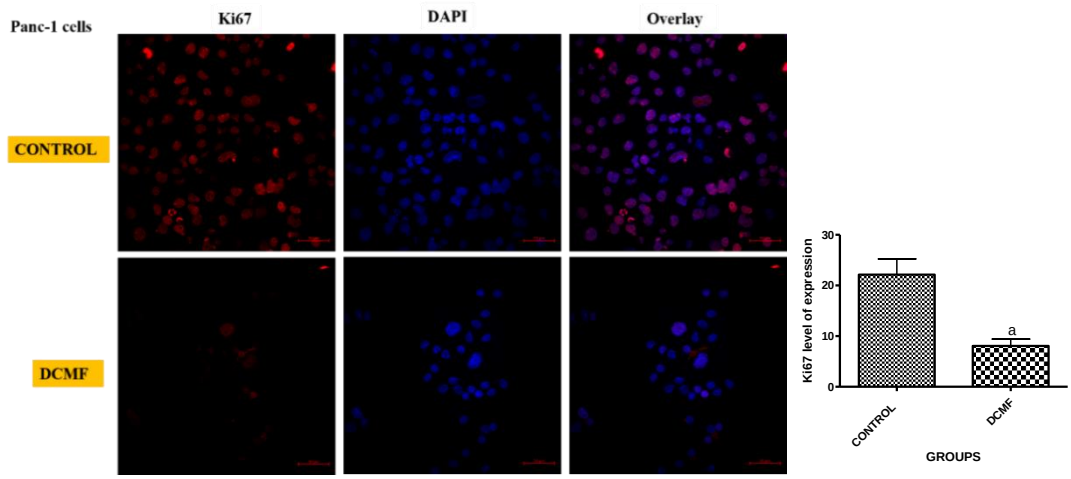


Figure 4.38. The dichloromethane fraction of *Tridax procumbens* downregulates Ki-67 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

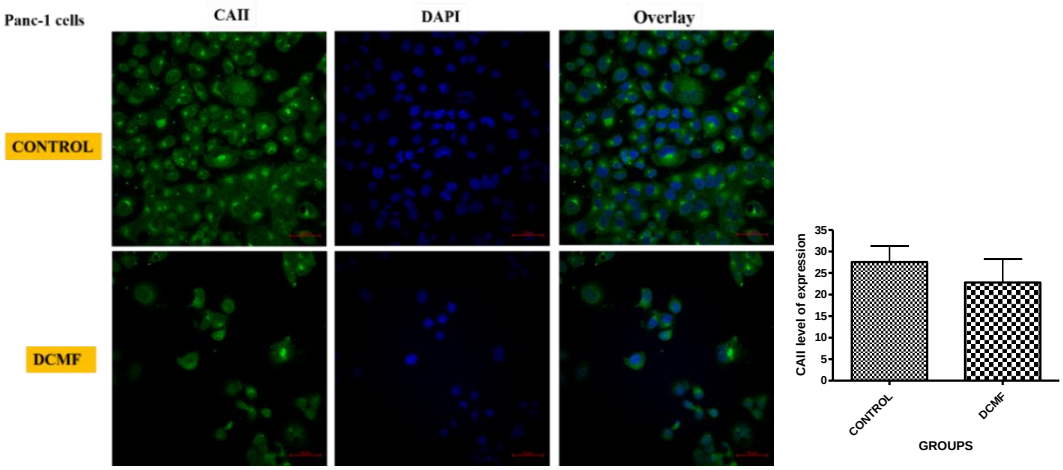


Figure 4.39. The dichloromethane fraction of *Tridax procumbens* reduced carbonic anhydrase II (CAII) activity after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

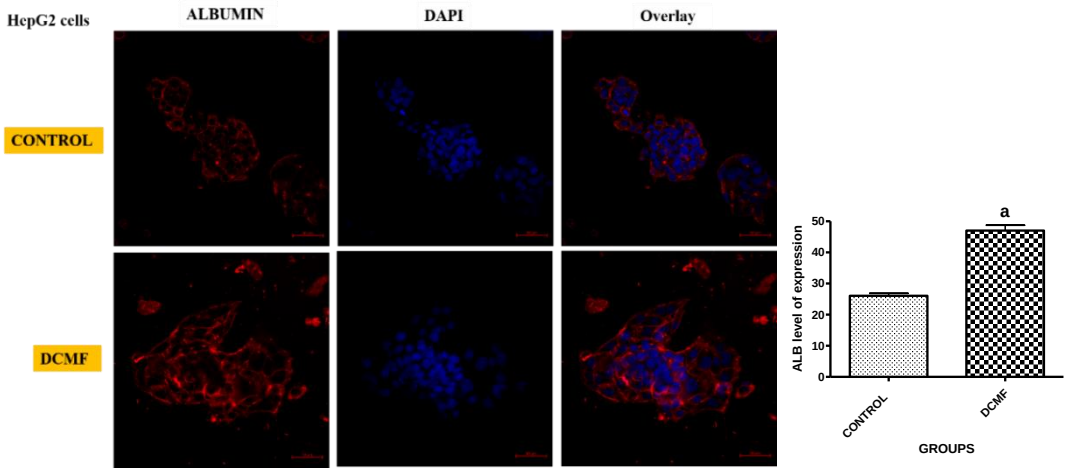


Figure 4.40. The dichloromethane fraction of *Tridax procumbens* upregulates albumin (ALB) in HepG2 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

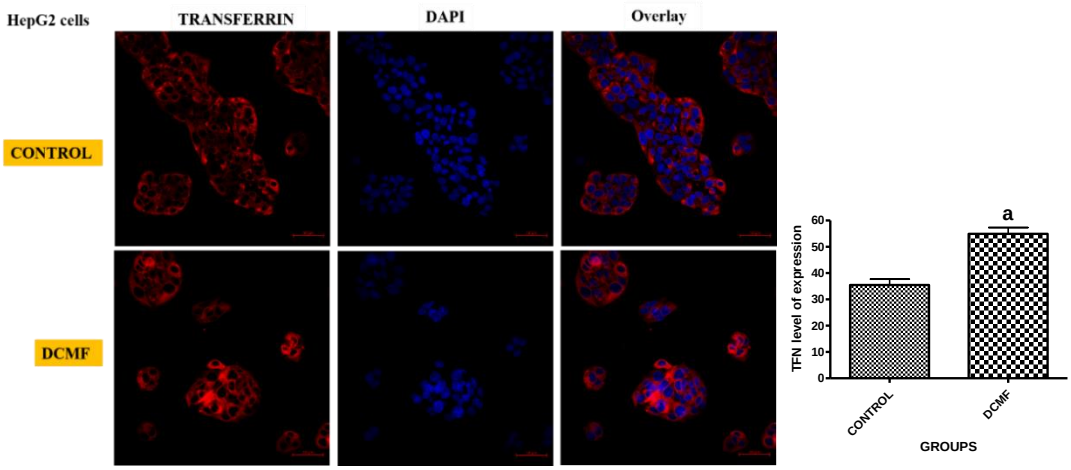


Figure 4.41. The dichloromethane fraction of *Tridax procumbens* upregulates transferrin (TFN) in HepG2 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

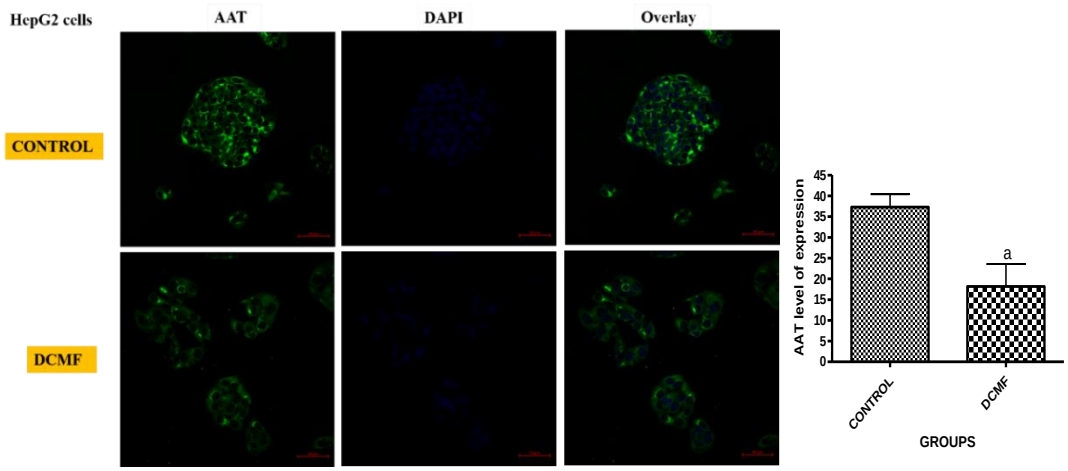


Figure 4.42. The dichloromethane fraction of *Tridax procumbens* downregulates α1-anti trypsin (AAT) in HepG2 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

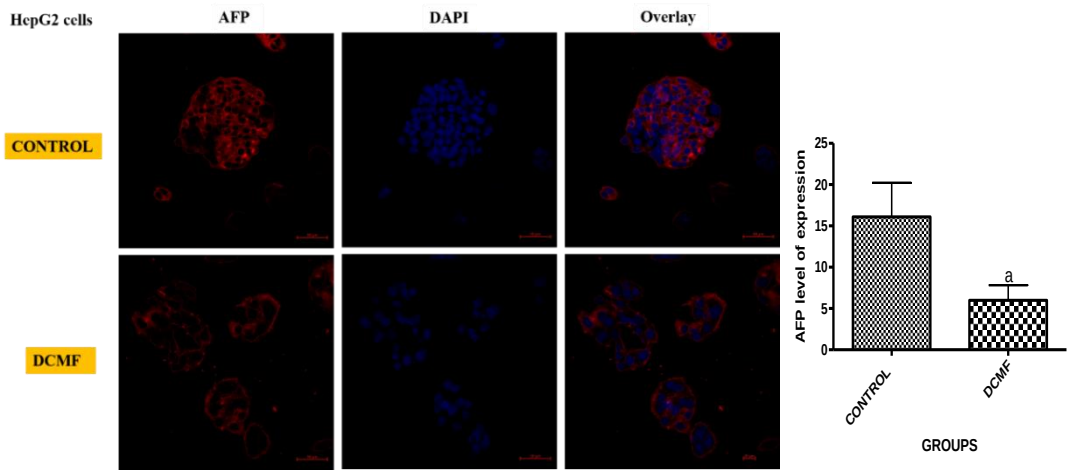


Figure 4.43. The dichloromethane fraction of *Tridax procumbens* downregulates alpha fetoprotein (AFP) in HepG2 cells after treatment (20 µg/ml) for 48 hours. [a] significantly different ($p < 0.05$) from the control. Control = dimethyl sulfoxide (DMSO), DCMF = dichloromethane fraction of *Tridax procumbens*. Scale bars are 50 µm and indicate the magnification. n = 3.

4.7. Objective 7: Isolation, purification, and identification of bioactive compounds from *Tridax procumbens* dichloromethane fraction using chromatographic and spectrometric techniques

The total sample yield of 1 g DCMF with column chromatography was 511.8 mg (51.2%) with subfractions D1 (98 mg 19.1%), D2 (136 mg, 26.6%), D3 (95 mg, 18.6%) and D4 (182.8 mg, 35.7%). D3 was selected based on potency in activities. The total sample yield of 90 g D3 with HPLC was 69.9 mg (77.7%) with subfractions D31 (33.1 mg/ml, 36.8%), D32 (11.5 mg/ml, 12.8%), and D33 (25.3 mg/ml, 28.1%). D32 was selected based on potency in activities. Total sample yield of 10 mg D32 with HPLC was 4.2 mg (42%) with isolates D321 (6/5 mg/ml, 12%), D322 (2/5 mg/ml, 4%), D323 (7/10 mg/ml, 7%), and D324 (4/5 mg/ml, 8%), D325 (11/10 mg/ml, 11%)

Five peaks with retention time in minutes (12.8, 14.2, 14.4, 15.3, 16.4) on HPLC chromatograms were identified (Figures 4.44 - 4.48) coupled with spectrum analysis of MS-MS showing fragmentation pattern (Figures 4.49 - 4.53). These peaks were identified and suggested to be Tsibulin 1, Pterosin N, 5,7,3'-Trihydroxy-2',4',5'-trimethoxyflavone, Corchorifatty acid D, and Capsiate, respectively (Figure 4.54). The characteristics of these bioactive compounds were also listed in Table 4.12.

Cytotoxicity was evaluated using MTT and Trypan blue assays on the Panc-1 cell line (Figures 4.55 - 4.57). Treatment with isolates ((20, 50 μg/ml) after 24 hours was done. It was noticed that the isolates from *Tridax procumbens* in a concentration-dependent manner attenuated Panc-1 cells viability (Figure 4.57). 1 g of the DCMF was partitioned with a column chromatography. Dichloromethane solvent with a gradient of methanol (0 - 20%) was used to elute the sample and this results in four fractions (D1 - D4). The fractions were assessed for purity using analytical thin-layer chromatography (TLC), and bands detected with a UV lamp (254 and 365 nm) and by spraying with sulphuric vanillin acid followed by heating (120 °C, 1 - 5 min). The fractions, D1 to D4, showed cytotoxic activities against Panc-1 cells with an inhibition rate of 34%, 62.19%, 96.45%, and 81.48%, respectively at 250 μg/mL using the MTT test (Figure 4.55).

However, Trypan blue test showed that the percentage viability of Panc-1 cells exposed to 20 μg/mL of D1 to D4 was 91.1, 94.8, 76.7, and 78.9, respectively. Based

on the TLC profile and the cytotoxicity test, D3 showed more activities and was considered for further separation by semi-preparatory HPLC which resulted in three fractions (D31 to D33). However, the fractions, D31 to D33, showed cytotoxic activities against Panc-1 cells with an inhibition rate of 12.27%, 57.16%, and 14.01%, respectively at 20 μg/mL using MTT assay (Figure 4.56).

Based on the TLC profile and the cytotoxicity test, D32 was considered for further purification using semi-preparatory HPLC which resulted in five isolated peaks (D321 to D325). However, the isolated peaks, D321 to D325, showed cytotoxic activities against Panc-1 cells with an inhibition rate of 31.35%, 64.70%, 40.26%, 40.43, and 38.83%, respectively at 50 μg/mL using the MTT test (Figure 4.57).

However, the level of p53 and p21 expression was also monitored in Panc-1 cells-treated isolates (Figures 4.60 and 4.61). The isolates especially isolate 2 (D322) and 4 (D324) caused upregulation of these proteins (Figures 4.60 and 4.61). Their pattern of staining on insulin, glucagon, amylase, and AFP was similar to that seen with dichloromethane fraction (Figures 4.58 and 4.59). However, there was more branching morphogenesis phenotype in isolates 2 (D322) and 4 (D324) (Figure 4.58).

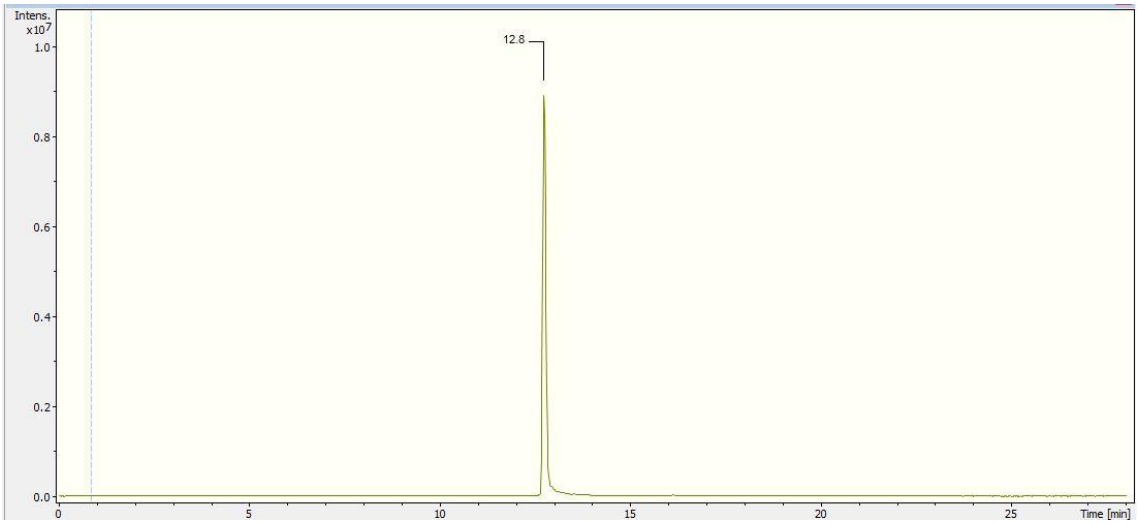


Figure 4.44. Spectrum analysis of LC-MS shows the retention time and relative abundance of the first isolate from the dichloromethane fraction of *Tridax procumbens.*

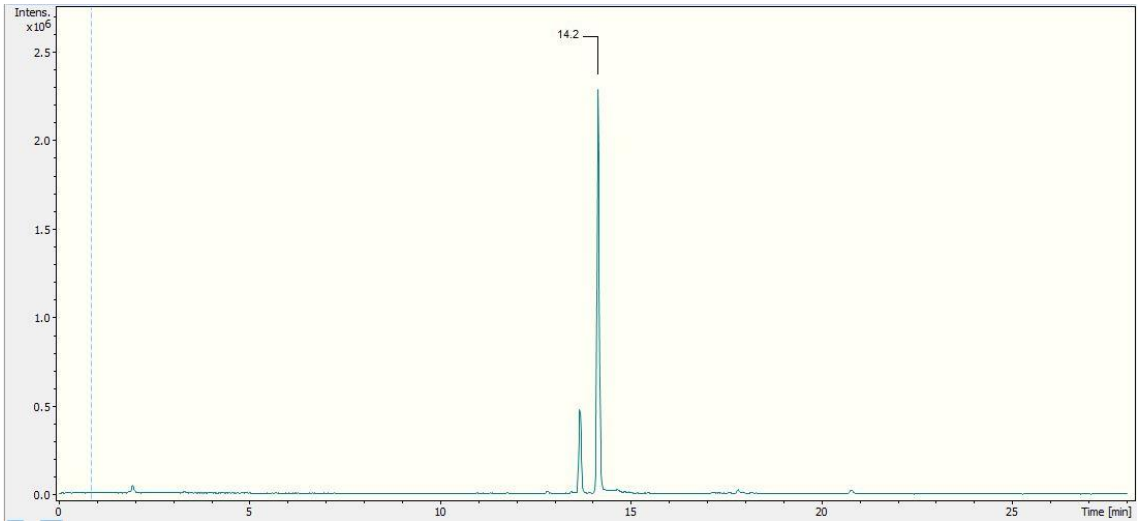


Figure 4.45. Spectrum analysis of LC-MS shows the retention time and relative abundance of the second isolate from the dichloromethane fraction of *Tridax procumbens.*

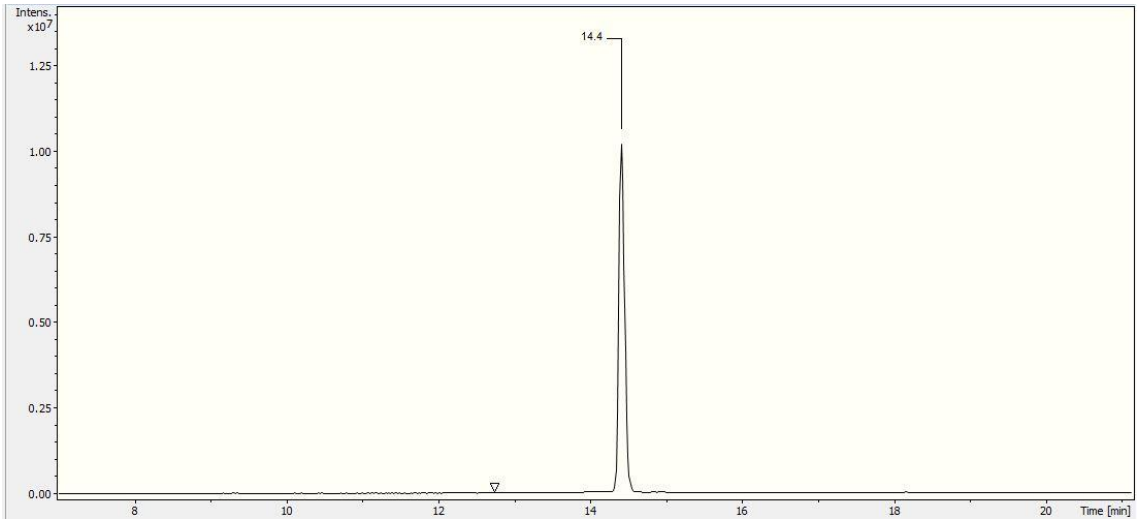


Figure 4.46. Spectrum analysis of LC-MS shows the retention time and relative abundance of the third isolate from the dichloromethane fraction of *Tridax procumbens.*

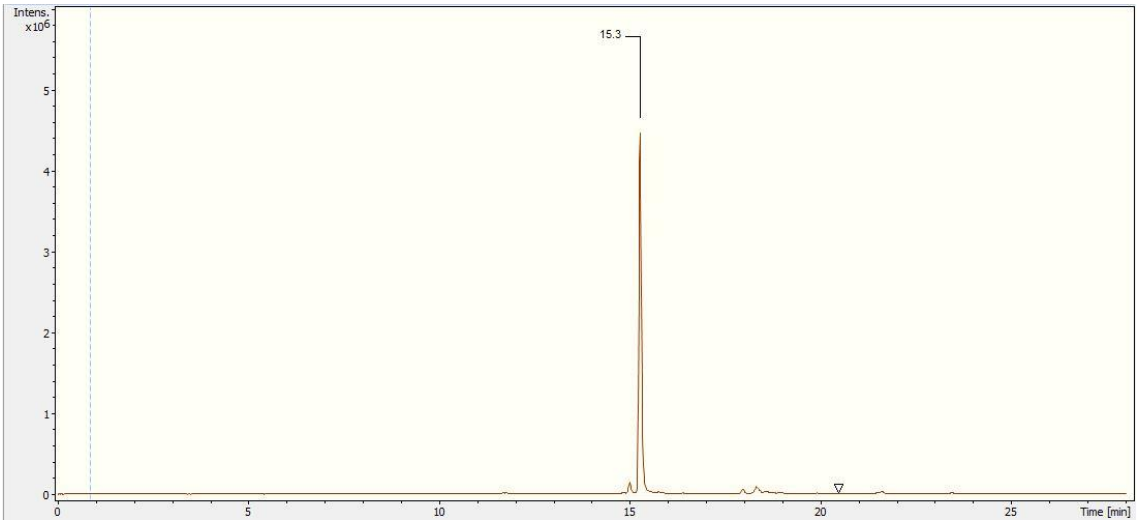


Figure 4.47. Spectrum analysis of LC-MS shows the retention time and relative abundance of the fourth isolate from the dichloromethane fraction of *Tridax procumbens*.

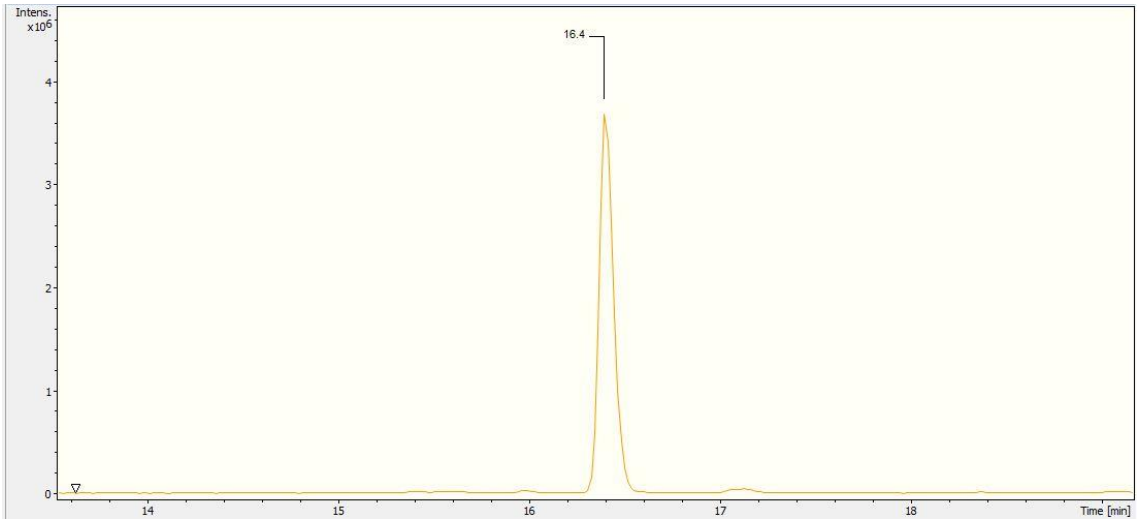


Figure 4.48. Spectrum analysis of LC-MS shows the retention time and relative abundance of the fifth isolate from the dichloromethane fraction of *Tridax procumbens.*

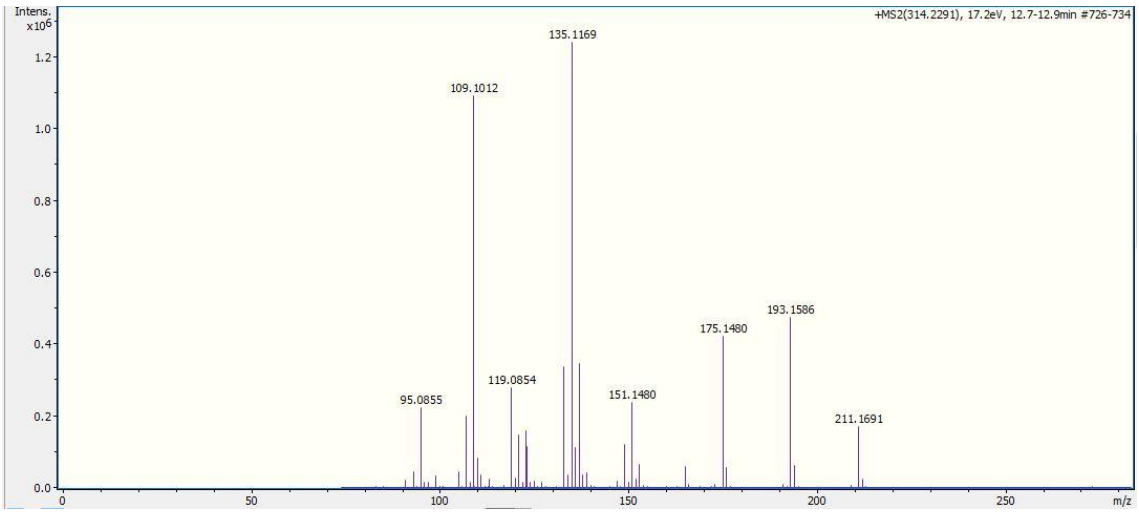


Figure 4.49. Spectrum analysis of MS-MS shows fragmentation arrangement of the first isolate from the dichloromethane fraction of *Tridax procumbens*.

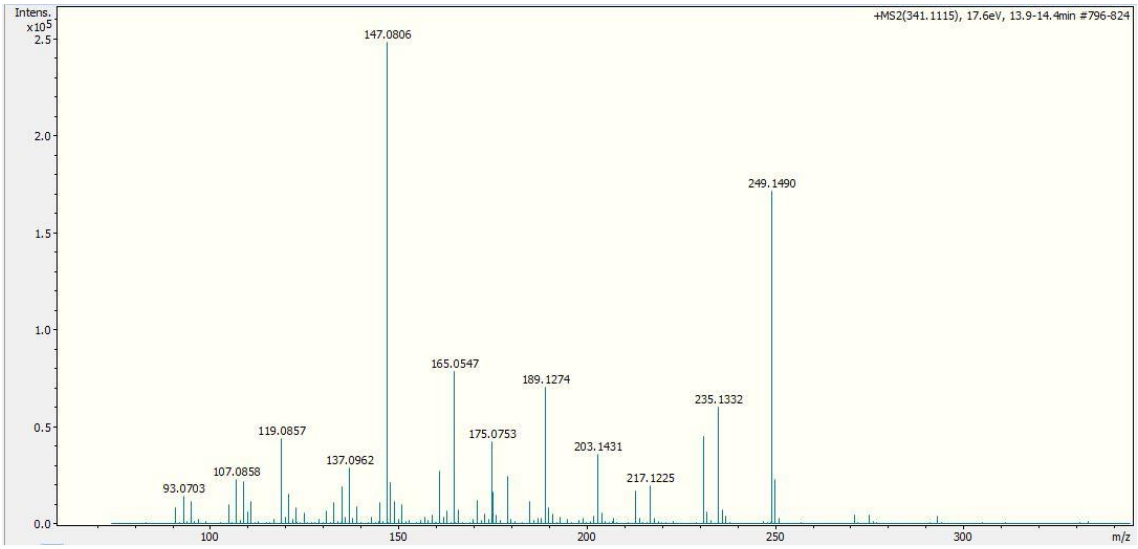


Figure 4.50. Spectrum analysis of MS-MS shows fragmentation arrangement of the second isolate from the dichloromethane fraction of *Tridax procumbens.*

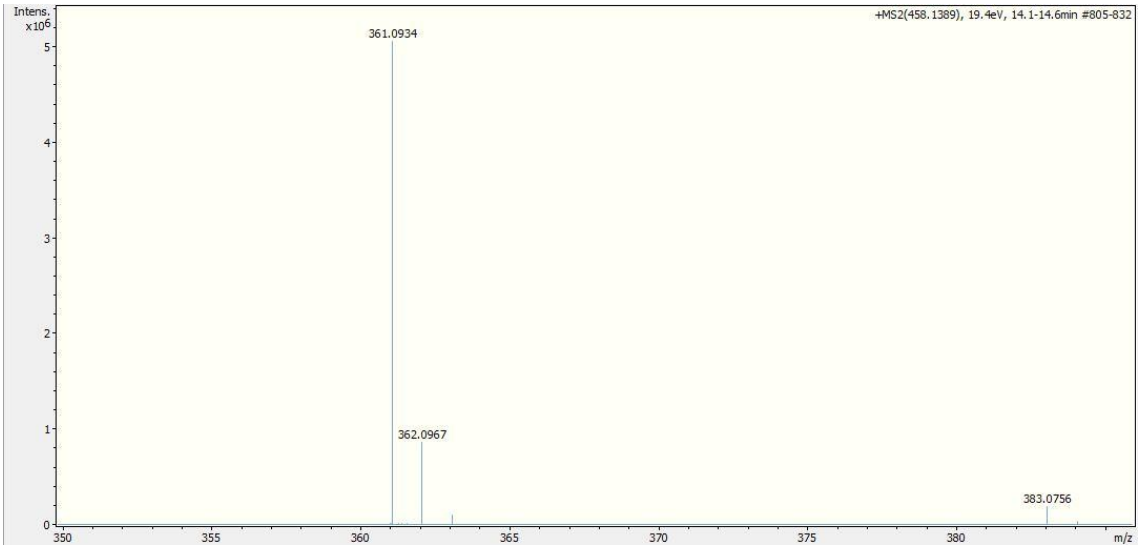


Figure 4.51. Spectrum analysis of MS-MS shows fragmentation arrangement of the third isolate from the dichloromethane fraction of *Tridax procumbens.*

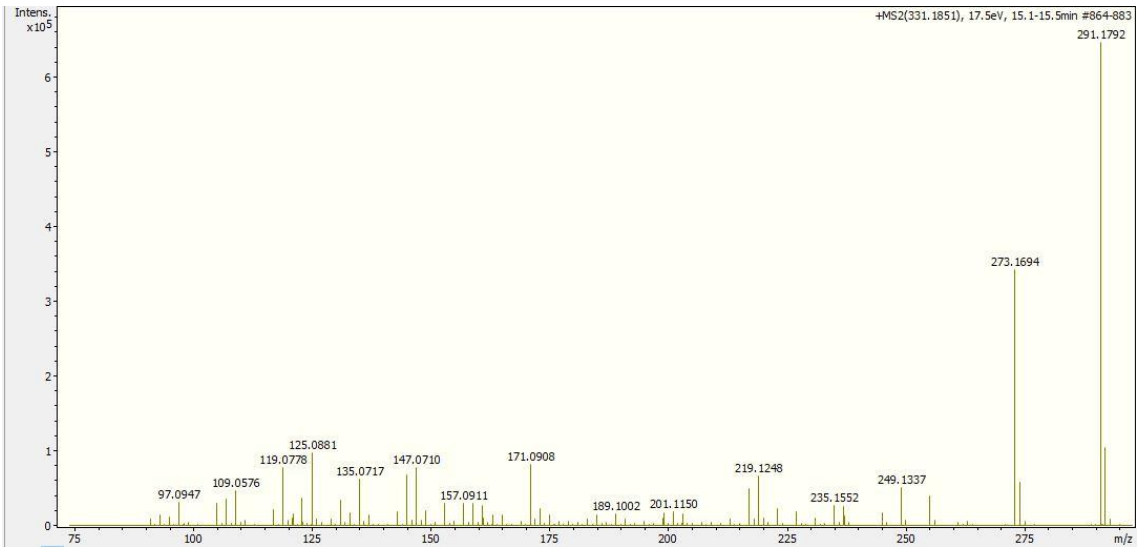


Figure 4.52. Spectrum analysis of MS-MS shows fragmentation arrangement of the fourth isolate from the dichloromethane fraction of *Tridax procumbens.*

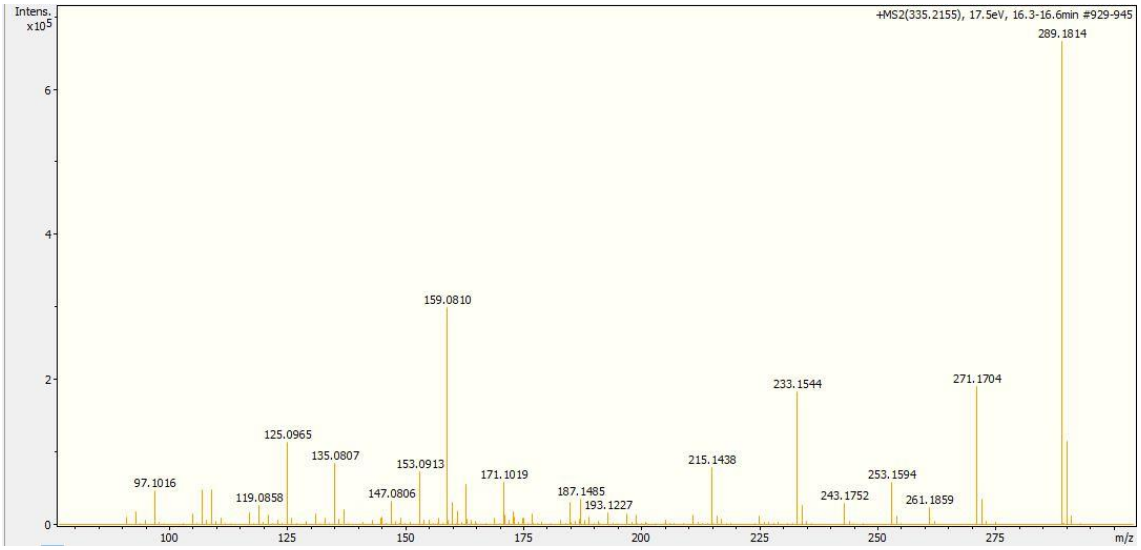


Figure 4.53. Spectrum analysis of MS-MS shows fragmentation arrangement of the fifth isolate from the dichloromethane fraction of *Tridax procumbens.*

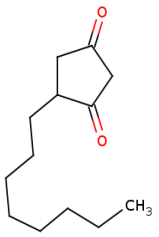
Table 4.12. Chromatographic and spectrometric characteristics of isolated and identified bioactive compounds from *Tridax procumbens*

S/N/Isolates	1	2	3	4	5
RT [min]	**12.8**	**14.2**	**14.4**	**15.3**	**16.4**
Area	**77510760**	**13294691**	**64168040**	**30184592**	**24793900**
m/z	**211.1691**	**235.1331**	**361.0916**	**309.2071**	**307.1916**
Ion Formula	**$C_{13}H_{23}O_2$**	**$C_{14}H_{19}O_3$**	**$C_{18}H_{17}O_8$**	**$C_{18}H_{29}O_4$**	**$C_{18}H_{27}O_4$**
Msigma	**1**	**15.1**	**18.2**	**23.1**	**17.6**
err (ppm)	**0.7**	**-0.8**	**0.4**	**-3.5**	**-3.9**
Referenced m/z	**211.1693**	**235.1329**	**361.0918**	**309.2060**	**307.1904**
Molecular Formula	**$C_{13}H_{22}O_2$**	**$C_{14}H_{18}O_3$**	**$C_{18}H_{16}O_8$**	**$C_{18}H_{28}O_4$**	**$C_{18}H_{26}O_4$**
Molecular weight	**210.162**	**234.1256**	**360.0845**	**308.1988**	**306.1831**

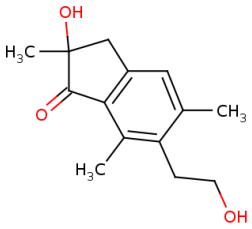
RT = Retention Time

m/z = mass to charge ratio

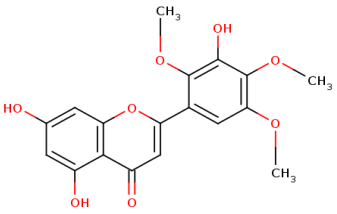
err = mass error



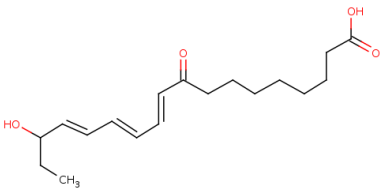
Tsibulin 1



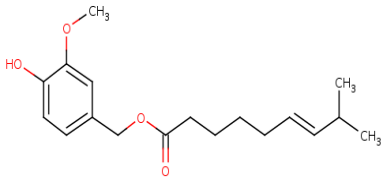
Pterosin N



5,7,3'-Trihydroxy-2',4',5'-trimethoxyflavone



Corchorifatty acid D



Capsiate

Figure 4.54. Structure of isolated and identified bioactive compounds from *Tridax procumbens*.

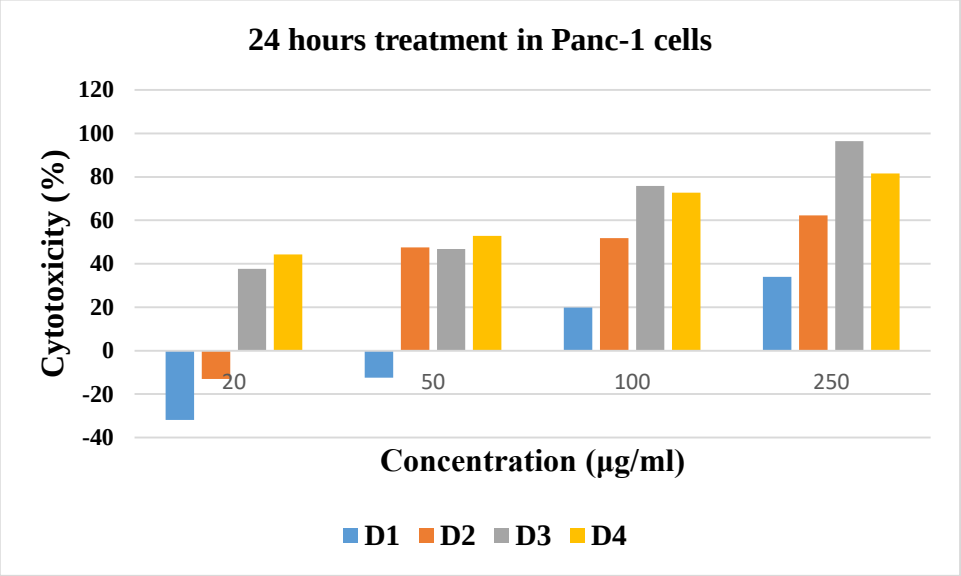


Figure 4.55. Subfractions of *Tridax procumbens* elicited Panc-1 cell death in a dose-dependent fashion using MTT assay after 24 hours of treatment. D3 fraction shows more cytotoxicity at increasing dosages. DI: fraction from dichloromethane and gradient of methanol (0 - 3%), D2: fraction from dichloromethane and gradient of methanol (4 - 5%), D3: fraction from dichloromethane and gradient of methanol (6 - 7%), D4: fraction from dichloromethane and gradient of methanol (8 - 20%). n = 3.

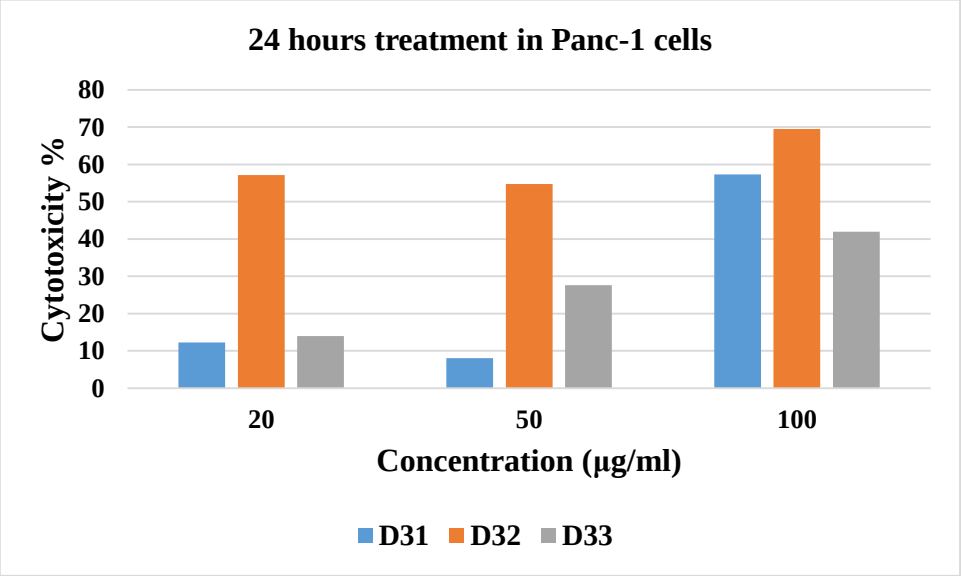


Figure 4.56. Semi-purified fractions of *Tridax procumbens* elicited Panc-1 cell death in a dose-dependent fashion using MTT assay after 24 hours of treatment. D32 fraction shows more cytotoxicity at increasing dosages. D3I: eluent of D3 collected from HPLC (0 - 17minutes of run time), D32: eluent of D3 collected from HPLC (17 - 21minutes of run time), D33: eluent of D3 collected from HPLC (21 - 35minutes of run time). n = 3.

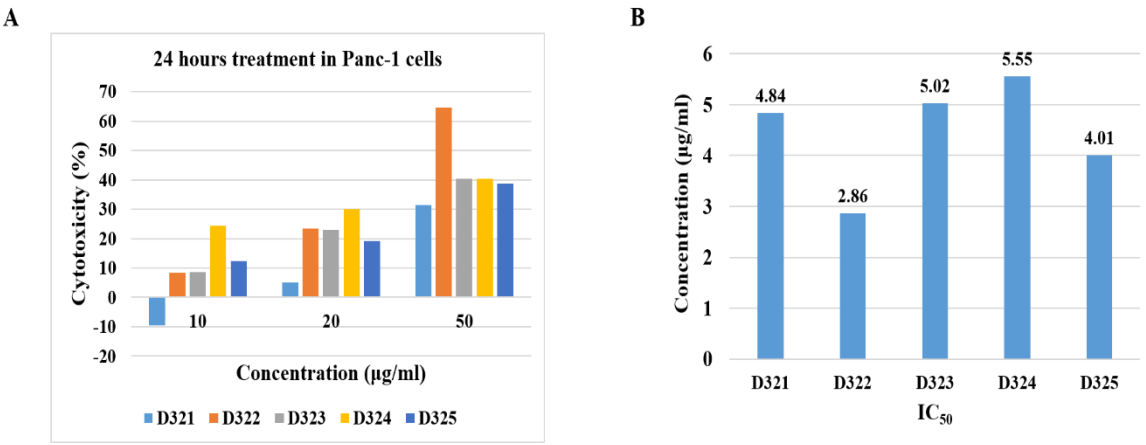


Figure 4.57. Identified bioactive compounds of *Tridax procumbens* elicited Panc-1 cell death in a dose-dependent fashion using MTT assay after 24 hours of treatment. D322 fraction shows more cytotoxicity at increasing doses. D32I - D325: five isolated peaks from sample D32. n = 3.

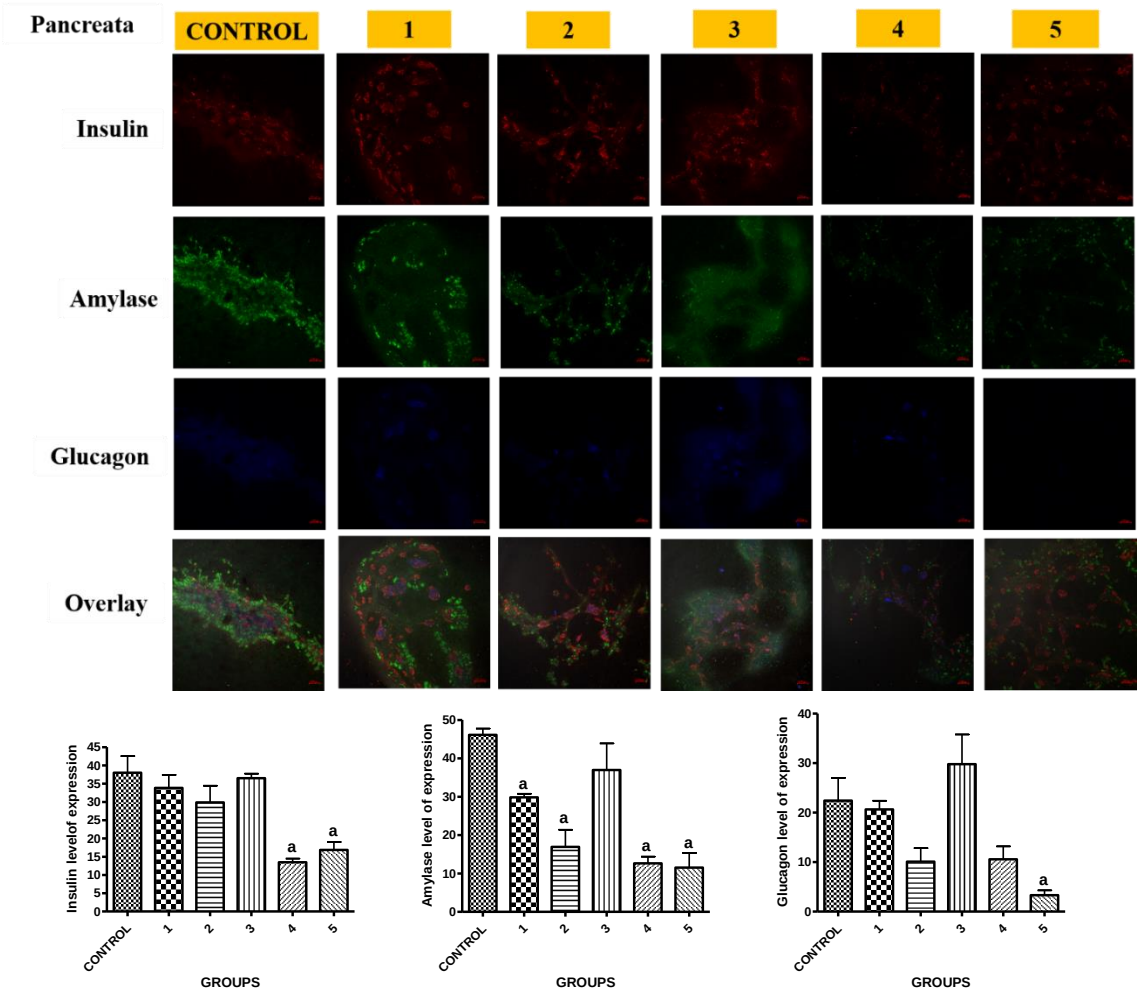


Figure 4.58. Identified bioactive compounds of *Tridax procumbens* stained positive for insulin, glucagon, and amylase in embryonic mouse pancreas after treatment (20 µg/ml) for 48 hours. 2 and 4 produced a phenotype of branching morphogenesis. Control = dimethyl sulfoxide. Isolates 1 - 5 (D321 - D325). Scale bars are 100 µm and indicate the magnification. n = 3.

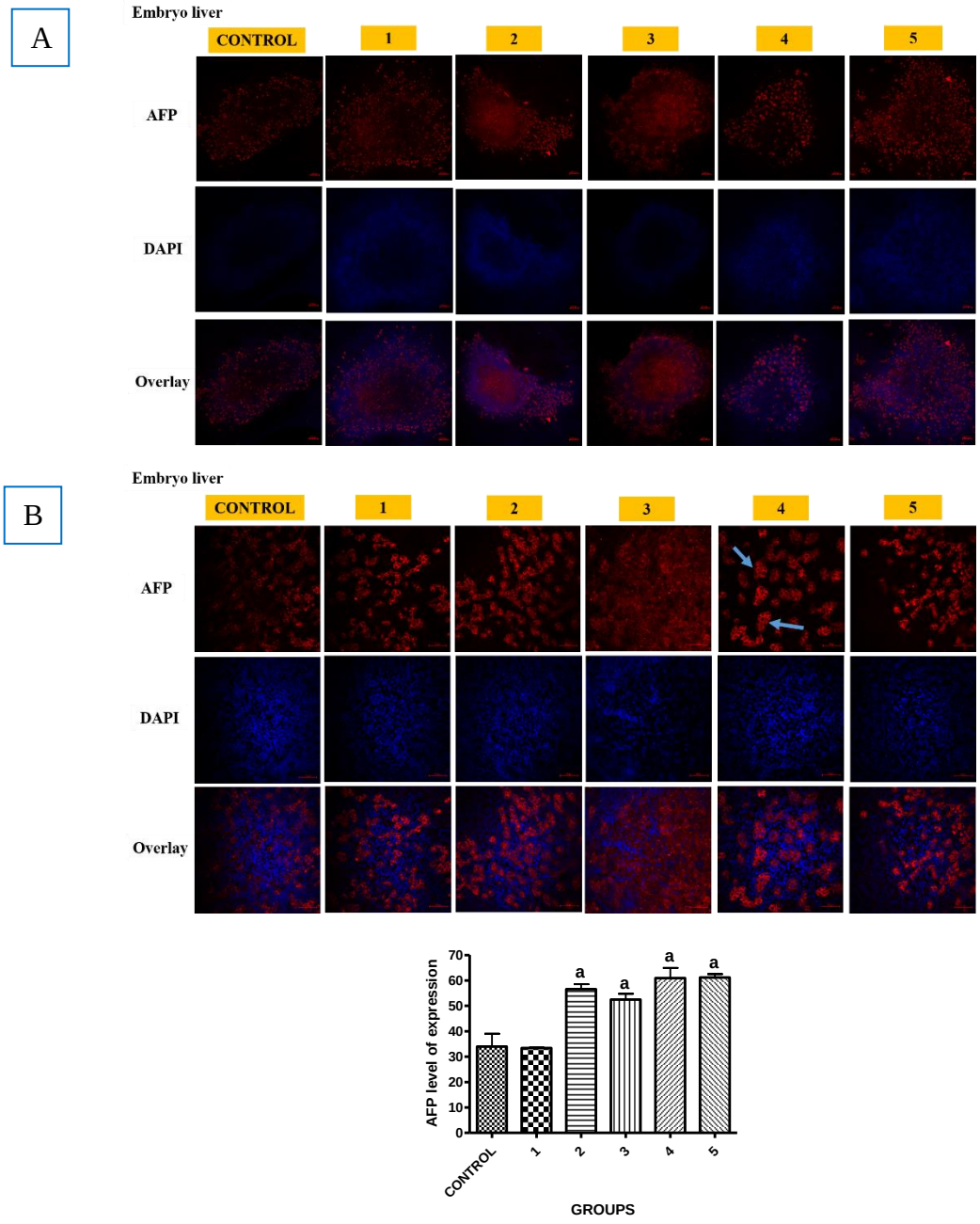


Figure 4.59. Identified bioactive compounds of *Tridax procumbens* stained positive for alpha-fetoprotein in embryonic mouse liver after treatment (20 µg/ml) for 48 hours (A). At higher magnification, it shows that 2 and 4 produced a phenotype of the hepatocytes forming strands and clusters in the embryonic mouse liver (B). Control = dimethyl sulfoxide. Isolates 1 - 5 (D321 - D325). Scale bars 100 µm (A) and 50 µm (B) indicate the magnification. n = 3

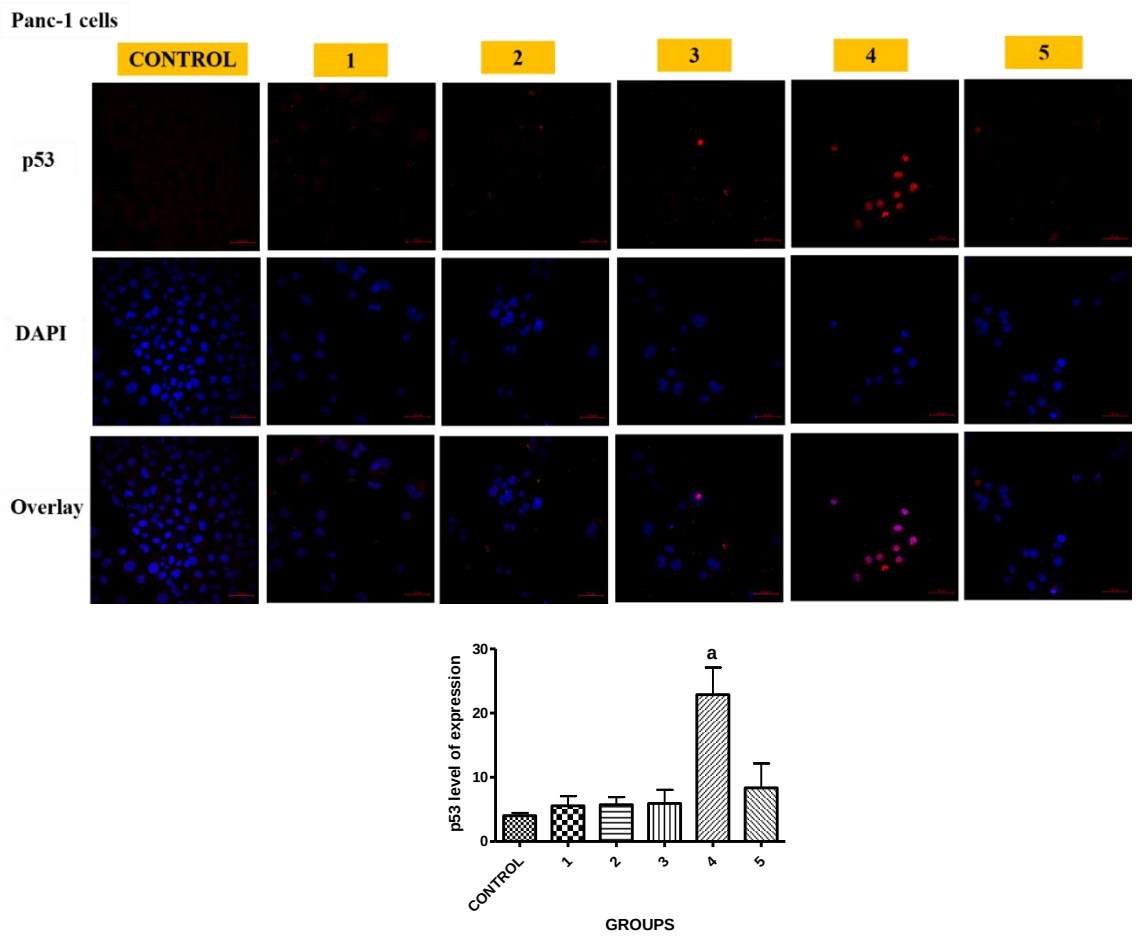


Figure 4.60. Identified bioactive compounds of *Tridax procumbens* upregulated p53 in Panc-1 cells after treatment (20 µg/ml) for 48 hours. Control = dimethyl sulfoxide. Isolates 1 - 5 (D321 - D325). Scale bars are 50 µm and indicate the magnification. n = 3.

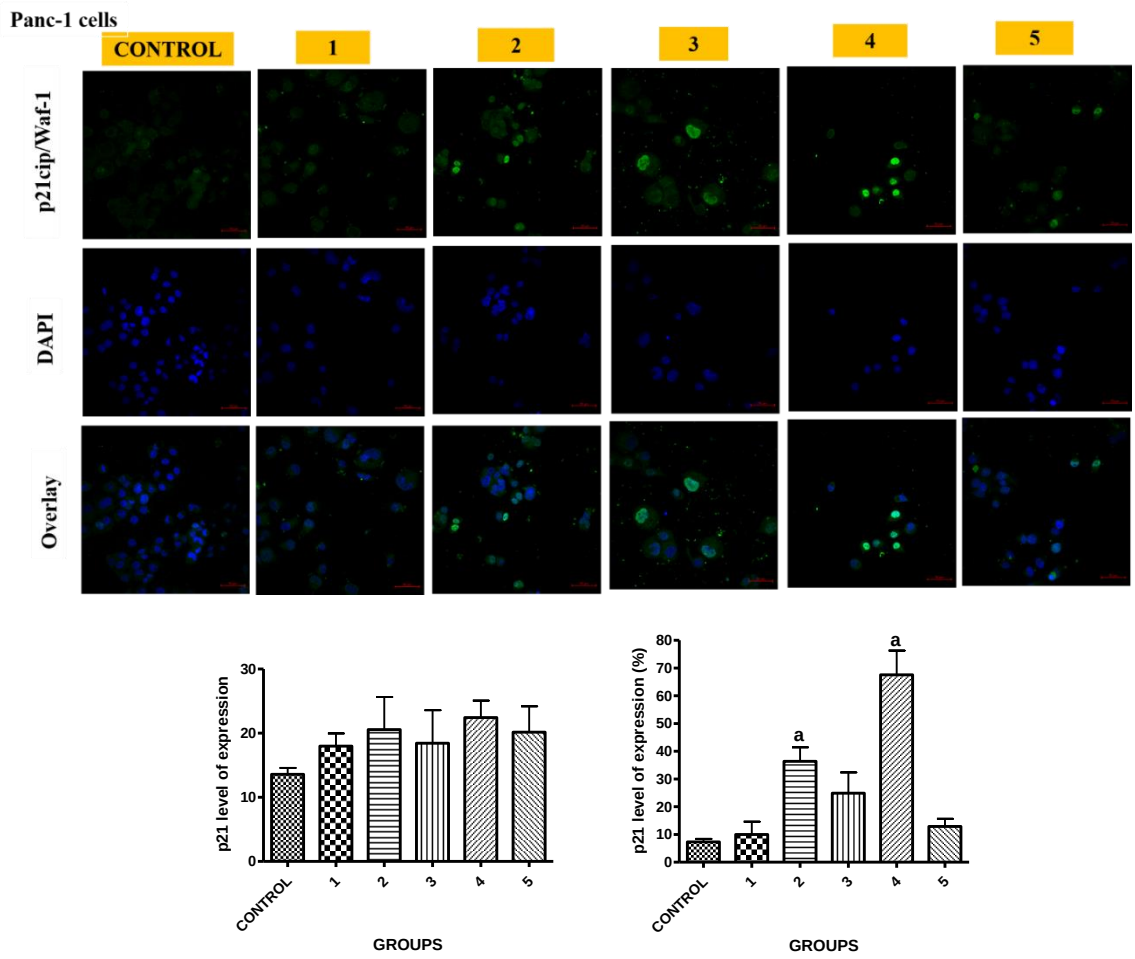


Figure 4.61. Identified bioactive compounds of *Tridax procumbens* upregulated p21$^{Cip1/Wap-1}$in Panc-1 cells after treatment (20 µg/ml) for 48 hours. Control = dimethyl sulfoxide. Isolates 1 - 5 (D321 - D325). Scale bars are 50 µm and indicate the magnification. n = 3.

CHAPTER FIVE

DISCUSSION

Research in medicinal plants has drawn a lot of scientific interest over the past years. Reason is that the use of medicinal herbs is a useful source of traditional medicines for treating different ailments such as malaria, and high blood pressure. However, one such plant is *Tridax procumbens,* a notable plant reported for its rich antioxidant phytochemicals, pharmacological values and uses in the management of high blood pressure, malaria, dysentery, haemorrhage, and wound (Talekar *et al.,* 2017).

5.1 *Tridax procumbens* contains phytochemicals with antioxidant activities

Phytochemical components were identified from *Tridax procumbens* in this study (Tables 4.1 and 4.11). Ethyl acetate followed by the dichloromethane fraction of *Tridax procumbens* has more antioxidant activities as compared to other fractions used (Figure 4.6), and this could have resulted from the observed high flavonoid and phenol content in the plant (Figure 4.5). This finding therefore could suggest the potential of *Tridax procumbens* leaf extract to modulate oxidative stress and oxidative stress-related disease conditions.

The observation from this study corroborate earlier reports (Singh and Ahirwar, 2010), therefore, suggesting the ability of ethanol extract of *Tridax procumbens* to mop up free radicals. Singh *et al.* (2017) stated that the ethanol extract of *Tridax procumbens* exhibited higher radical scavenging activities in comparison with both its methanol and aqueous extracts. Agrawal *et al.* (2009) documented that, n-butanol and ethyl acetate fractions of *Tridax procumbens* possess comparable antioxidant activities on DPPH. However, this observation contradicts the statements of Sunil *et al.* (2012), that documented the lack of flavonoids, saponins, and tannins in the ethanol extract of *Tridax procumbens*.

5.2 *Tridax procumbens* leaf extract modulates dysfunctions mediated by sodium arsenite in rats

Observation from this study shows that *Tridax procumbens* extract caused a non-significant bodyweight change in the rats (Figure 4.1). This suggests that *Tridax procumbens* extract could not elicit deleterious alteration in the body and organ weights of selected organs in this experiment and justify the statement of Preuss *et al.* (2002). There was a report of a non-significant bodyweight change property of *Tridax procumbens* by Keerthi *et al.* (2014) when they administered the ethanol extract of *Tridax procumbens* (250 and 500 mg/kg bwt) to their experimental animal model.

Interestingly, Petchi *et al.* (2013) had earlier reported that *Tridax procumbens* whole-component ethanol extract did not affect animals' body weight. However, this is slightly contrary to the reported weight-reducing potential of *Tridax procumbens* (Ikewuchi and Ikewuchi, 2009c) and some plant extracts (Ezekwesili-Ofili and Gwacham, 2015). It was said that body weight reduction by plant extract may be due to the activation of beta-3 cellular receptors which elicit thermogenesis, and subsequently increased lipid hydrolysis and metabolic rate (Preuss *et al.*, 2002).

The findings on haematology in this study (Table 4.4) showed that the *Tridax procumbens* extract at the dosage used could not reverse the haemotoxicity induced by sodium arsenite in the test rats. These corroborate the documentation of Bhagwat *et al.* (2008) that reported a non-significant reduction in haemoglobin value in animals treated with *Tridax procumbens*. Moreover, Ikewuchi (2012) and Ikewuchi *et al.* (2013) reported increased RBC counts, PCV, and haemoglobin concentration in animals treated with *Tridax procumbens*, and that this could be linked to its high Fe composition (Ikewuchi and Ikewuchi, 2009b).

The elevation in the platelet count of the group treated with extract only may suggest the anti-haemorrhage activity of the *Tridax procumbens* as stated by Ali *et al.* (2001) and Ikewuchi (2012). The pattern of leukocytosis caused by sodium arsenite in this study could be compared to what has been seen in cases of stress and poisoning (Odutola, 2000). According to Odutola, (2000), sharp elevation in white blood cell counts mainly occurs during inflammation as one of the first lines of the body system's

defence against infection, poisoning, and stress (Odutola, 2000). Also, the decrease in leukocyte count (Table 9) after the administration of *Tridax procumbens* extract may be related to its usage against inflammation and stress. This follows the contribution of Swenson and Reece (1993) that, noxious plants have no direct effect on white blood cell counts including their differentials.

However, it was reported in some studies that WBC performs crucial roles in the cardiovascular system (Takeda *et al.*, 2003). These studies corroborate the report that increased WBC counts in peripheral blood are a familiar predisposing factor to blood vessel diseases (Takeda *et al.*, 2003). Thus, the reported increased WBC counts caused by arsenite in the present study suggest and support the report of Tiwari *et al.* (2004) that categorised arsenic as a predisposing factor in blood vessel disease.

The result from this present research showed that *Tridax procumbens* extract at the dosage of 50 mg/kg could not reverse sodium arsenite-mediated hepatotoxicity and therefore inhibit protein biosynthesis (Figure 4.2). This finding corroborates the documentation of Abubakar *et al.* (2012b) but is however contrary to reports of Hemalatha (2008) and Ikewuchi (2012b) that stated the protective activity of *Tridax procumbens* on the liver at different concentrations.

It is worth knowing that some medicinal plants were earlier said to exhibit hepatotoxic properties due to elevation in clinical chemistry proteins such as total protein, albumin, ALT, AST, ALP, and GGT (Ranjna, 1999). These proteins are however released into the bloodstream from the cytosol and subcellular organelles in significant amounts after damage or acute injury to the liver (Lu, 1996). Serum albumin has been reported to be a good index to monitor the secretory capability of hepatocytes (Yakubu *et al.*, 2005). The non-significant difference in the value of creatinine and BUN seen in this study (Table 4.7) supports the documentation of Jude and Catherine, (2013) on *Tridax procumbens*.

Findings in the current research suggested that *Tridax procumbens* could be utilised in managing diabetes mellitus-linked complications such as hyperlipidaemia, and preventing defects in lipid metabolism. This function may relate to the occurrence of flavonoids and tannins in *Tridax procumbens* but this claim warrants further

investigation. There are reports of antihyperlipidaemic activity of *Tridax procumbens* (Petchi *et al.,* 2013; Keerthi *et al.,* 2014).

The mean percentage motility and sperm count or concentration of all the groups were reduced significantly in the present study (Figure 4.8). These values were above 60% which could suggest, according to Zemjanis (1970) that the extract may not be able to cause infertility at the dosage used but could be toxic. In addition, Zemjanis (1970) documented that for an animal to be grouped or categorised satisfactorily regarding breeding soundness examination, spermatozoa motility and viability should not be fewer than sixty per cent. Hafez (1993) stated in his study that a high spermatozoa motility and viability percentage will lead to high fertility potential.

Ola-Davies *et al.* (2014a) reported the potential anti-fertility of the plant used in their study because the reported sperm motility and livability percentages were fewer than 60%. Furthermore, the observed sperm characteristics across the groups were within the usual range of ten to twenty per cent (Bishop *et al.,* 1949; Zemjanis, 1970; Hafez, 1993), and with no significant variation in the means values among the groups for both testicular and epididymal morphometry (Table 4.9 - 4.10).

This does not support the previous report of Olayemi *et al.* (2011) on *Cnestis ferruginea*. A reduction in both testicular and epididymal weight and length following administration of methanol fraction of *Spondias mombin* was documented (Ola-Davies *et al.,* 2014b). These derangements could interfere with the sperm storage; transport and maturation function of the epididymis (Olayemi *et al.,* 2011).

Findings from this present study (Figure 4.4) thus suggest that *Tridax procumbens* extract can ameliorate the clastogenic effect of sodium arsenite and by inference possible anticancer activities. This statement corroborates the findings of Adewale *et al.* (2013) that used a similar dose of methanol extract of some plants. Meanwhile, micronuclei numbers were reported to be an index for predicting the carcinogenic ability of chemicals (Celik *et al.,* 2005).

Arsenic could cause chromosomal damage and stimulate micronuclei formation with most scientists not yet certain if it can induce direct gene mutation (Goyer, 1991;

Odunola, 2003; Aliyu *et al.*, 2012; Owumi *et al.*, 2012). This vivid pardon, together with an occasional poor comparison between exposure to arsenic, dose, and resultant relative frequency of chromosomal dysregulation was clarified with the hypothesis that arsenic enhances gene dysfunctions greatly by preventing DNA repair (Bencko *et al.*, 1988). This inhibition may be a fundamental pathway for the mutagenicity and assumably the carcinogenicity of arsenic (Okui and Fujiwara, 1986).

The arsenite-induced histological lesions observed in the lungs, brain, and testes in the present work (Plate 4.1 - 4.6) could relate to the reported pathologic changes usually elicited by arsenite because of its potential to trigger oxygen metabolites production and glutathione reduction (Lee *et al.*, 1989; Lee and Ho, 1995). *Tridax procumbens* was able to reduce the severity of the lesions (Plate 4.1 - 4.6).

This amelioration ability of *Tridax procumbens* corroborates the reports of earlier scientists that plants such as *Juglans nigra*, and *Peucedanum grande* could protect the tissue architecture of experimental animals from damages mediated by chemicals (Owumi *et al.*, 2013; Adewale *et al.*, 2019). This ability did not apply to the liver as *Tridax procumbens* could not ameliorate the damage induced by arsenite. Similar findings have been reported by Abubakar *et al.* (2012b).

5.3 *Tridax procumbens* induces differentiation of mouse embryonic pancreas and enhances the population of liver progenitor cells

Pancreatic tissue constitutes two unique categories of cells called endocrine and exocrine pancreatic cells. The former is located in the islets of Langerhans that spreads across the latter and consists of 5 cell types namely; alpha, beta, delta, pancreatic polypeptide, and the fifth cell type that produces glucagon, insulin, somatostatin, polypeptide, and ghrelin (Broglio *et al.*, 2003; Murtaugh and Melton, 2003). The cell types all function to control metabolism.

On the other hand, the exocrine portion consists of both the acinar which secrete amylase and the ductal cells. Research documentation showed that the dorsal and ventral primordia develop into the endocrine, exocrine, and ductal cells that in combination constitute the pancreatic tissue (Slack, 1995; Kim and Hebrok, 2001).

Percival and Slack, (1999) and Gu *et al.* (2002) produced strong shreds of evidence that the same endodermal rudiment produces the endocrine and exocrine cells.

The developing embryo liver emanates from the anterior foregut endoderm and the mesenchyme of the septum transversum (Ward and Tosh, 2010). This hepatic fate takes place at around embryonic day 8 (E8), and fibroblast growth factors and bone morphogenetic growth factor 4 play crucial roles in the correct specification of the liver (Jung *et al.,* 1999; Rossi *et al.,* 2001; Duncan, 2003). Following a period of multiplication and growth (~E9.0-9.5), at E11.5, the embryonic liver becomes the biggest visible organ within the abdomen of the embryo, and the liver haematopoiesis is also well formed.

Several days after E15.5, the intestine and liver are linked via the bile duct (Lemaigre, 2003). In this study, markers of the various pancreatic (Figures 4.18 - 4.22), hepatic (Figure 4.17) and intestinal (Figure 4.24) cells, respectively were monitored. There was increased expression of alpha-fetoprotein in the crude extract, dichloromethane, and ethyl acetate fractions-treated liver tissue (Figure 4.17A). At higher magnification, the test samples, especially the dichloromethane fraction, produced a phenotype of the hepatocytes forming strands (Figure 4.17B).

In addition, there was immunopositivity for insulin, glucagon, somatostatin, amylase, cytokeratin 7, peanut agglutinin, and vimentin in the extract and fractions treated groups (Figures 4.18 - 4.22). However, it was observed that there was enhanced branching morphogenesis of the ductal system in the DCMF-treated cultured embryonic pancreas based on increased expression of cytokeratin 7 and peanut agglutinin (Figures 4.20 and 4.21).

These findings showed that *Tridax procumbens* especially the dichloromethane fraction does not inhibit differentiation in both the exocrine and endocrine pancreas. This is contrary to the previous study where retinoic acid, a member of the vitamin A family inhibits exocrine differentiation and suppresses morphogenesis in the pancreas (Shen *et al.,* 2007). However, two underlying mechanisms of exocrine differentiation inhibition that have been suggested; enhanced secretion of laminin and activation of apoptosis (Shen *et al.,* 2007).

In their study, apoptotic markers such as Bax, and stain like propidium iodide were used to determine if their test sample was inducing apoptosis (Tulachan *et al.*, 2003). There are also conflicting reports regarding the relative effects of retinoic acid on pancreatic differentiation (Kadison *et al.,* 2001; Chen *et al.,* 2004).

In addition, results from the current experiment have proved that *Tridax procumbens* especially dichloromethane fraction enhanced the population of AFP-expressing hepatoblasts (bipotent liver precursors that generate hepatocytes and ductal cells) and could not cause cell death. These findings could be relevant in an attempt to produce the various cell types of the pancreas and liver in transplantation. The observed increase and decrease in percentage contraction of the DCMF-treated oesophagus and intestine respectively might be a mechanism of action of the plant (Figure 4.23).

5.4 *Tridax procumbens* fraction suppresses Panc-1 and HepG2 cells via markers of oxidative stress, apoptosis, proliferation, differentiation and metastasis

The downregulation of GST-pi by dichloromethane fraction of *Tridax procumbens* in this study (Figure 4.43) suggests its ability to abrogate oxidative stress, and potentially serve as an anticancer agent. Reports have it that GST-pi is expressed in physiological human epithelial tissues (Sarkar *et al.,* 1977; Terrier *et al.,* 1990). Moreover, there are suggestions that the increase in GST-pi activity correlates with malignant transformation, resistance to anticancer agents, and poor prognosis (Ribrag *et al.,* 2003) while its downregulation in rat hepatoma cells favours apoptosis (Asakura *et al.,* 2001).

Meanwhile, one of the widely distributed isoenzymes in the body system is the Glutathione *S*-Transferases (GST) family that has varying isoforms (alpha, delta, kappa, mu, pi, sigma, theta, zeta). They protect healthy cells from cytotoxic agents and carcinogens, and participate in the phase II detoxification process that involves glutathione (Mannervik *et al.,* 1985).

It has been shown in this study that the dichloromethane fraction of *Tridax procumbens* upregulates catalase activity (Figure 4.31). This buttresses the point that *Tridax procumbens* could be beneficial against oxidative stress and by inference in

cancer management. Hydrogen peroxide is one of the reactive species derived from oxygen. Due to its slow reaction kinetics with most biomolecules, hydrogen peroxide can build up within cells hence, transforming into other very reactive species that can become toxic or lead to oxidative stress. However, catalase, one of the two fundamental cellular antioxidant enzymes (Li *et al.*, 2011a) participates in cell defence against oxidative stress (Glorieux *et al.*, 2015) through the dismutation of $2H_2O_2$ into $2H_2O$ and O_2.

It has been documented that catalase also performs decomposition (Kono *et al.*, 1998; Heinzelmann and Bauer, 2010), oxidation (Brunelli *et al.* 2001), marginal peroxidase (Johansson and Borg, 1988), and low oxidase activity (Doskey *et al.*, 2016). Meanwhile, there was a report of reduced activity of this enzyme in malignant cells (Lewis *et al.*, 2005) while several therapeutic agents increased its activities (Suzuki *et al.*, 2000; Klingelhoeffer *et al.*, 2012).

In this present study, *Tridax procumbens* treatment increased alkaline phosphatase levels in Panc-1 cells (Figure 4.30). This observation is comparable to the effect of all-*trans*-retinoic acid on alkaline phosphatase activity in Panc-1 cells (Guo *et al.*, 2006). Also, *Tridax procumbens* treatment was reported to induce primary osteoblasts differentiation with significantly increased alkaline phosphatase activity (Al Mamun *et al.*, 2017).

Alkaline phosphatase (ALP) is recognised as a crucial index of differentiation in human malignancies (Dabare *et al.*, 1999; Perán *et al.*, 2013). The initiation of differentiation of human pancreatic cancer by a specific inhibitor of DNA methylation (MIA PaCa-2 cells) promotes ALP expression (Yamada *et al.*, 1996). In their study, they likened increased expression of alkaline phosphatase after treatment with test samples to enhance differentiation in a cancer cell line.

There were earlier reports of several polyphenolic compounds especially quercetin inhibiting proliferation and also inducing differentiation biomarkers like ALP (Lenhard *et al.* 2000; Lea *et al.*, 2010). Meanwhile, synergistic elevation in its activity was reported in colon cancer cells after simultaneous treatment with butyrate and MAPK signalling inhibitors (Witt *et al.*, 2001; Lea *et al.*, 2007). The reduction in

GST-pi and elevation in both alkaline phosphatase and catalase activities in DCMF-treated cells suggest the potential antioxidant capability of *Tridax procumbens* in addition to its ability to enhance the differentiation of cancer cells. This ability could be due to the presence of the flavonoid and phenol in the sample (Figure 4.5).

The present study demonstrated that *Tridax procumbens* induced p53 (Figure 4.25) and p21 (Figure 4.26) levels, and by inference suggests that it could stop the cell cycle, hinder multiplication, cause DNA damage, and stimulate cancer cell death. To confirm this, the expression levels of apoptotic markers and cytotoxicity tests were investigated. The induction of caspase-3 (Figure 4.27), elevation in the perinuclear and nuclear punctate staining of Bcl-2 (Figure 4.28), coupled with the significant cytotoxicity (Figures 4.7 - 4.12) in the cancer cells treated with dichloromethane fraction of *Tridax procumbens* supports this hypothesis.

p53 is a well-studied tumour suppressor protein (Muller and Vousden, 2014; Chen *et al.*, 2017) that detects stresses encountered by cells. The protein protects the cells via various biochemical pathways (Ribas *et al.*, 2015). Alteration of p53 results in the loss of its wild-type functions often detected in many different tumour types (Muller and Vousden, 2014; Chen *et al.*, 2017).

This tumour suppressor protein has been reported to perform its functions through activation of a transcription target p21; whose one of its major roles is G1-S/G2-M phase impairment (Dulic *et al.*, 1998). p53-dependent p21 gene transcriptional activation occurs following DNA injury (El-Deiry *et al.*, 1993). p53/p21/CDK regulatory phase is an important mode of action for DNA damage-mediated arrest of the cell cycle. Abrogation of p53 signalling pathways is believed to be required for the growth of most malignant cells, and there is evidence to show that restoration of p53 function has a huge therapeutic benefit (Muller and Vousden, 2014).

Chemotherapeutic agents have been said to cause some degree of DNA damage to cancer cells which can result in the rapid upregulation of p53 protein levels by a posttranscriptional mechanism (Harris, 1996; Gewirtz, 2000). The eventuality of the upregulation of p53 could lead to either apoptosis or arrest of the cell cycle. Caspase-3 was reported to participate in the morphological and biochemical changes of apoptotic

cells with several nuclear targets despite the cytoplasmic localisation of pro-caspase 3 (Earnshaw *et al.,* 1999). Also, Bcl-2 is an apoptotic inhibitor with reports suggesting that its nuclear localisation promotes apoptosis in nature (Bryce and Giulio, 2006). Thus, it could be argued that the eventuality of the upregulation of p53 and p21 proteins in this study was through the apoptotic pathway.

There was a translocation of Adenomatous polyposis coli's (APC) protein in the dichloromethane fraction of *Tridax procumbens* treated-Panc-1 cells from nuclear to cytoplasmic localisation (Figure 4.32). This could translate to the enhancement of the tumour suppressor activity of APC, consequently, the abrogation of cell proliferation biomarkers such as beta-catenin, Sox9, and Ki-67. The APC main function is to control β-catenin (Sena *et al.,* 2006). APC is said to be a negative regulator of both beta-catenin and the Wnt signalling pathway in an activity that takes place in the cytoplasm (Korinek *et al.,* 1997).

Adenomatous polyposis coli with cytoplasmic loci in normal physiological states have nuclear localisation in some cancer cell types (Sena *et al.,* 2006). APC together with other members of the complex downregulates the Wnt signalling and beta-catenin (Korinek *et al.,* 1997; Eisinger *et al.,* 2006). When there is dysregulation of APC, beta-catenin will link up with TCF/LEF leading to Wnt downstream target effectors upregulation hence, cell proliferation stimulation (Eisinger*et al.,* 2006).

The current study showed that the dichloromethane fraction of *Tridax procumbens* downregulated beta-catenin (Figure 4.33) and Sox9 (Figure 4.34) in HepG2 cells and caused a cytoplasmic translocation of the proteins after 48 hours of treatment. Also, the dichloromethane fraction of *Tridax procumbens* downregulated Ki-67 in the Panc-1 cell (Figure 4.38). The observed downregulation and cytoplasmic expression of beta-catenin (Figure 4.33) and Sox9 (Figure 4.34) in treated HepG2 cells in the present study together with the significant downregulation of Ki-67 and the translocation of APC in Panc-1 cells (Figure 4.32) might be that one of the mechanistic actions of *Tridax procumbens* is mitigation of the Wnt/beta-catenin cascade.

β-catenin is an important cellular protein and transcriptional factor that contributes to development under normal physiological conditions (Wodarz and Nusse, 1998; Lien

and Fuchs, 2014). Beta-catenin participates in Wnt signalling aside from being involved in cellular regeneration and renewal (Lien and Fuchs, 2014). Dysregulation of β-catenin can activate the malignant transformation of normal cells resulting in many cancer types (Morin, 1999; Polakis, 2000). In a normal physiological state (absence of the ligand Wnt), the cytoplasmic accumulation of beta-catenin is controlled and kept low via a complex of protein inhibitors that comprises of APC/GSK-3β/axin (van Es *et al.,* 2003; Nelson *et al.,* 2004).

This complex performs a continuous phosphorylation-induced ubiquitin-mediated degradation of β-catenin thereby preventing its movement into the nucleus (Dajani *et al.,* 2003). However, Wnt signalling stimulation will result in GSK-3β phosphorylation, consequently APC/GSK-3β/axin complex dissociation. This will prevent β-catenin degradation and allows for its cytoplasmic accumulation, and eventually, its nuclei translocation.

This process allows β-catenin to interact and activates several downstream transcription factors. β-catenin also controls the transcription of various effector proteins that are linked to cell survival, growth, proliferation, and invasion (Dajani *et al.,* 2003; Jeong *et al.,* 2012).

Also, the Sox protein family has been implicated strongly in human developmental processes where they are involved in differentiation, lineage restriction regulation, and cartilage formation (Lefebvre *et al.,* 2007). However, notable among the Sox proteins is Sox9, a transcription factor. Sox9 promotes differentiation in the testis coupled with cartilage formation and is also expressed in the hepatocytes during hepatocyte regeneration (Emai *et al.,* 2014). However, there are reports of the overexpression of Sox9 in cancer of the liver and breast (Guo *et al.,* 2012), colon (Lu *et al.,* 2008), and pancreas (Grimont *et al.,* 2015).

These have been associated with cancer invasiveness, aggressive characteristics, and bad prognosis (Lu *et al.,* 2008). This protein is recorded to be a downstream substrate of the Wnt signalling and together with β-catenin controls differentiation (Blache *et al.,* 2004).

Besides, the Ki-67 protein has been said to be a cellular marker associated strictly with proliferation (Scholzen and Gerdes, 2010; Genc *et al.*, 2018) and exclusively detected during interphase within the cell nucleus and relocated to the surface of the chromosomes in mitosis (Genc *et al.*, 2018). Reports have it that Ki-67 expression in cells indicates tumour multiplication rates and this corresponds with initiation, progression, invasion (Lee 1996; Genc *et al.*, 2018). Agreement between this protein proliferation index and neuroendocrine tumours concerning tumour size, biologic behaviour, and angioinvasion was documented (Rindi *et al.*, 2006, 2007; Genc *et al.*, 2018a).

There is, however, a recent study that describes the use of Ki-67 proliferation index to estimate the postoperative recurrence of pancreatic neuroendocrine tumours (Genc *et al.*, 2018b). The reduction in the expression of vimentin (Figure 4.35) and CK7 (Figure 4.36) in Panc-1 cell treated with *Tridax procumbens* in this study suggest the plant's inhibitory role on resistance to apoptosis, increased cellular migratory capacity and tumour metastasis or invasiveness. The developmental process called epithelial-mesenchymal transition allows mature polarised epithelial cells to go through changes biochemically and take up a mesenchymal phenotype of resistance to apoptosis, increased invasive capacity, and expression of extracellular matrix factors (Beuran *et al.*, 2015).

The process is termed dedifferentiation and was said to be linked with an elevation in the expression of mesenchymal markers or cell-specific proteins such as vimentin, and a decrease in the expression of epithelial markers like E-cadherin (Cates *et al.*, 2009). Also, cytokeratin 7 (CK7), a subtype of the intermediate filament protein called cytokeratin has been characterised. For example, CK7 is well described in normal tissues (Rafiee *et al.*, 1992) and implicated in some forms of malignancies (Johnson *et al.*, 1988, Hruban and Fukushima, 2007).

The reduced level of AFP (Figure 4.43) and AAT (Figure 4.42) in HepG2 cells treated with dichloromethane fraction of *Tridax procumbens* in this study further confirms the inherent ability of *Tridax procumbens* to inhibit cell proliferation and metastasis. Bosetti *et al.* (2014) reported that liver cancer is not only an emerging form of cancer but it is the 5th commonest cancer and the 3rd most found cause of cancer-associated

deaths globally considering a five-year survival rate is 7%. Nearly half of the estimated 750,000 liver cancer cases diagnosed worldwide in 2008 were seen in China (Farrell *et al.*, 2010).

However, there are reports of well-characterised biomarkers such as AFP (α-fetoprotein) and AAT (α-1-antitrypsin) used to detect hepatocarcinoma (Johnson *et al.*, 2001; Li *et al.*, 2001). AFP level in the fetal serum is usually elevated but decreased to a significant level after birth similar to that of an adult. This is due to the reduced ability to produce AFP in the adult liver (Wang and Wang, 2018). There is, however, a regain of the ability to produce and express AFP by liver cells with malignant transformation (Buendia and Neuveut, 2015; Gao *et al.*, 2016).

Meanwhile, AAT, the most found serpin (Li *et al.*, 2017) is exclusively synthesised by the liver cells (70 - 80%), with other sources like macrophages and intestinal epithelia reported (Geboes *et al.*, 1982; Boskovic *et al.*, 1998). In normal physiological conditions, AAT protects the lungs from excess uninhibited neutrophil elastase (Li *et al.*, 2017). However, there are reports of elevated amounts of AAT in cancer patients (Comunale *et al.*, 2010) and its *de novo* production was documented in malignancy (Higashiyama *et al.*, 1992) together with its involvement in the distant invasiveness of lung adenocarcinoma (Zelvyte *et al.*, 2004). AAT carries out this function via fibronectin upregulation (Li *et al.*, 2017).

The dichloromethane fraction of *Tridax procumbens* downregulated the carbonic anhydrases II (CAII) level after 48 hours of treatment (Figure 4.39) in the present study. This portrays the differentiation ability of the fraction and this could cause a possible DNA damage to be repaired or channel towards apoptosis. However, in the current study, it is put forward that apoptosis will be the eventuality as was observed with the upregulation of apoptotic markers such as caspase-3 and the punctate perinuclear staining of the Bcl-2 protein. Carbonic anhydrases are a class of enzymes with 14 isoforms. They speed up the hydration reaction of CO_2 and also control acid-base equilibrium in a variety of tissues (Carter 1972) in addition to their tissue-specificity and varying subcellular localisations.

However, the activities of CAII were well reported (Kumpulainen and Jalovaara 1981; Parkkila *et al.,* 1994). This enzyme is a terminal differentiation marker of the pancreatic duct with huge involvement in pancreatic juice secretion that contains a high amount of bicarbonate (Mahieu *et al.,* 1994), and its expression is inducible by a differentiating agent such as retinoid (Frazier *et al.,* 1990; Tarek *et al.,* 2005).

In the current study, the dichloromethane fraction of *Tridax procumbens* downregulates peanut agglutinin (Figure 4.37). This could be one of the mechanisms of the actions of the plant by regulating the glycosylation changes associated with peanut agglutinin. Meanwhile, the contemporary trend in research highlights a focus on lectins, a group of proteins that preferentially recognise and with great accuracy bind complexes of carbohydrates. They do this in a way that cells are agglutinated through polysaccharides and glycoconjugates precipitation (Rüdiger and Gabius, 2001).

Some lectins have also performed anticancer functions (De Mejía and Prisecaru, 2005; Fu *et al.,* 2011). However, a protein of 110 kDa MW called Peanut agglutinin (PNA) has attracted researchers' interest, especially in cancer development (Banerjee *et al.,* 1994). Peanut agglutinin has both anti-malignant (Kiss *et al.,* 1997; Mukhopadhyay *et al.,* 2014) and malignant activities (Singh *et al.,* 2006; Zhao *et al.,* 2014). This protein possesses high T-antigen specificity as a result of its lack of any carbohydrate moiety. However, abnormalities in glycoconjugate are usually associated with epithelial malignant neoplasm and their precursors like inflammatory bowel disease and colonic polyps (Campbell *et al.*, 2001).

Also, invasion and metastasis in tumour cells have been linked to these abnormalities (Hakomori, 2001). The abnormalities in the expression of carbohydrate moiety in cancer types such as colon cancer include Thomsen-Friedenreich (TF) elevated expression (Campbell *et al.,* 1995). A commonly found glycosylation alteration in several pre-cancerous and cancerous epithelial is TF overexpression (Campbell *et al.,* 1995; Rhodes *et al.,* 2008). TF is the native ligand of human galactoside-binding galectin-3 (Yu *et al.,* 2007). Dysregulation of galectin-3 expression encourages cancer progression and invasiveness through its association with various galactoside-terminated cell surface glycans (Liu *et al.,* 2005; Newlaczyl *et al.,* 2011). Peanut

agglutinin interaction with TF can induce mitosis in neoplastic cells, and promotes cell proliferation in both normal and diseased states (Ryder *et al.*, 1992, 1994).

In the present study, there was an upregulation of albumin (Figure 4.40) and transferrin (Figure 4.41) levels after treating HepG2 cells with a dichloromethane fraction of *Tridax procumbens*. This could be attributed to the differentiation capacity of the dichloromethane fraction of *Tridax procumbens* and its potential to suppress the proliferation of hepatocarcinoma cells indirectly via the upregulation of these proteins. Several models of hepatocyte-specific transcription have been studied, and the serum albumin gene is one of them.

However, the albumin gene is selectively expressed in hepatocytes and this was seen during liver bud formation in the mouse embryo, the foetus, and in the mature liver (Liu *et al.*, 1988; Cascio and Zaret, 1991). There is a decrease in serum albumin levels (hypoalbuminaemia) in cancer patients due to the increasing demand for amino acids by the proliferating tumour mass, which is fulfilled by digesting the available albumin (Bairagi *et al.*, 2015). Hypoalbuminaemia is characteristic of patients with advanced solid tumours, hence albumin-based drug delivery systems would be profitable (Hauser *et al.*, 2006; Cho *et al.*, 2008).

Moreover, several conditions are characterised by hypoalbuminemia, hence the need for upregulation in albumin levels (U.S. Patent, 2016). Upregulating albumin expressions inhibit tumour development and growth in cancerous/hyper-proliferative cells, respectively (U.S. Patent, 2016). Albumin transcripts activation in HepG2 cells represses proliferation indirectly. This inhibition was proposed to be indirect, in that, albumin does not involve in cell proliferation like Ki-67 or p21 but the upregulation of albumin will divert resources from cell proliferation and eventually stop or reduce proliferation.

Inference can be drawn from this function of albumin to transferrin. Transferrin, a Fe-transport protein, is produced in the hepatocytes (Morgan, 1981). However, because of this property, transferrin could be referred to as a growth factor (Richardson and Ponka, 1997). There is also evidence that other cells like T4 lymphocytes and Sertoli cells produced transferrin, and this can allow for specialised multiplication and

differentiation (Skinner and Griswold, 1980). Moreover, there are reports of the inductions of differentiation functions of neoplastic cells through the upregulation of albumin and transferrin after treatment with cell differentiating agents (Herman *et al.,* 1981).

In this study, five (5) peaks were isolated (Figures 4.44 - 4.48), identified (Figures 4.49 - 4.53), and characterised (Table 4.12) from the dichloromethane fraction of *Tridax procumbens*. These peaks are suggested to be Tsibulin 1, Pterosin N, 5,7,3'-Trihydroxy-2',4',5'-trimethoxyflavone, Corchorifatty acid D, Capsiate (Figure 4.54). It was demonstrated in this study that these compounds especially Pterosin N and Corchorifatty acid D could upregulate the activity of p53 (Figure 4.60) and p21 (Figure 4.61), consequently stopping the cell cycle and stimulating apoptosis.

Furthermore, the pattern of staining for markers specific for the pancreas (Figure 4.58) and liver (Figure 4.59) by the compounds especially Pterosin N and Corchorifatty acid D suggests that they do not inhibit differentiation in the liver and pancreas unlike previous study (Shen *et al.*, 2007). Therefore, our findings have proved that the isolated compounds from *Tridax procumbens* could not induce cell death in tissue explants.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

6.1 Summary

The widespread environmental contamination by arsenic and the incessant rise in global cancer incidence has increased the search for better preventive and treatment interventions. The usefulness of medicinal plants as one of the alternative therapies can not be overemphasised. One of such plants with potential therapeutic efficacy is *Tridax procumbens* L. (Asteraceae) that has been employed in the management of wounds, malaria, high blood pressure, stomach ache, bronchial catarrh, and as feed in animals that could be prone to arsenic exposure.

The lack of information on the effect of *Tridax procumbens* in pancreatic ductal adenocarcinoma, hepatocellular carcinoma, and arsenite-induced toxicity using *in vivo* and *in vitro* models that warranted this study has been addressed. In this study, *Tridax procumbens* ameliorated arsenite-induced dysfunctions in rats, was cytotoxic to Panc-1 and HepG2 cells, but not toxic to embryonic mouse tissues. These activities were suggested to be due to the presence of antioxidant phytochemicals in the *Tridax procumbens* extract as found in the study.

6.2 Conclusions

This study shows that *Tridax procumbens* is rich in phytochemicals and has free radical scavenging activity. This might be the reason for the observed amelioration effects of *Tridax procumbens* on the dysfunctions mediated by sodium arsenite in the cells of the lungs, testes, brain, and bone marrow of the Wistar rats. 50 mg/kg bwt *Tridax procumbens* administered to the rats could not ameliorate the haemotoxicity, spermatotoxicity, and hepatotoxicity induced by sodium arsenite.

The dichloromethane fraction of *Tridax procumbens* used in this study was the most potent fraction with significantly higher cytotoxic activity on Panc-1 and HepG2 cells.

The dichloromethane fraction of *Tridax procumbens* downregulated the expression of CK7, PNA, vimentin, Sox9, beta-catenin, AFP, AAT, CAII, GST-pi, and Ki-67, and upregulated the expression of catalase, ALP, caspase3, APC, p53, and p21Cip1/Wap-1 in the cell lines. This suggests the ability of *Tridax procumbens* to abrogate cell proliferation, tumour development, and metastasis.

The elicited branching morphogenesis in the pancreatic bud and formation of hepatocytes strands by the dichloromethane fraction evident with the proportion of cells positive for CK7 and PNA and AFP, respectively suggests the ability of *Tridax procumbens* not to mediate cell death in normal physiological conditions. Also, the staining pattern for markers specific to the pancreas and liver suggests that *Tridax procumbens* did not inhibit differentiation in the embryonic tissues at normal physiological conditions.

Five peaks suggested to be Tsibulin 1, Pterosin N, 5,7,3'-Trihydroxy-2',4',5'-trimethoxyflavone, Corchorifatty acid D, and Capsiate were characterised from dichloromethane fraction of *Tridax procumbens*. It was demonstrated that these compounds also retained the properties of the dichloromethane fraction of *Tridax procumbens* in the experimental models used. Therefore, it is suggested that *Tridax procumbens* modulates sodium arsenite-mediated dysfunctions in Wistar rats, and indices of growth, proliferation, and invasiveness in pancreatic ductal adenocarcinoma and human hepatocellular carcinoma cell lines and mouse embryo.

Tridax procumbens carried out its activities through suppression of clastogenicity, oxidative stress, Wnt signaling, cell cycle, EMT pathway, and metastasis. Also, it stimulated apoptosis in the cancer cells, induced differentiation of pancreatic endocrine and acinar cell types, and enhanced the population of AFP-expressing hepatoblasts (bipotent liver precursors that generate hepatocytes and ductal cells).

6.3 Recommendations

1. *Tridax procumbens* should be listed together with the promising medicinal plants utilised in pancreatic ductal adenocarcinoma and hepatocellular carcinoma management.

2. The elucidation of the mechanisms of action of the bioactive compounds from *Tridax procumbens* in embryonic tissues should be investigated.

6.4 Contributions to the knowledge

1. This is most likely the first study to report the protective ability of *Tridax procumbens* leaf extract on sodium arsenite-mediated dysfunctions in the brain, bone marrow, testes, and lungs of male Wistar rats at the dosage used together with its cytotoxic effect on Panc-1 and HepG2 cell lines.
2. This study is probably the first report to show that *Tridax procumbens* induced nuclear/cytoplasmic translocation of GST-pi, Bcl-2, Sox9, beta-catenin, APC proteins in Panc-1 and HepG2 cell lines.
3. This is most likely the first study to show the antiproliferative effect of *Tridax procumbens* in Panc-1 and HepG2 cells via the induction of caspase-3, p53, p21, increased antioxidant enzyme system and decreased Ki-67, vimentin, cytokeratin-7, beta-catenin, and Sox9 proteins.
4. This study is probably the first to show the inability of *Tridax procumbens* to elicit cell death in embryonic mouse pancreas and liver, and its ability to induce differentiation of pancreatic endocrine and acinar cell types, and enhance the population of alpha-fetoprotein-expressing hepatoblasts.

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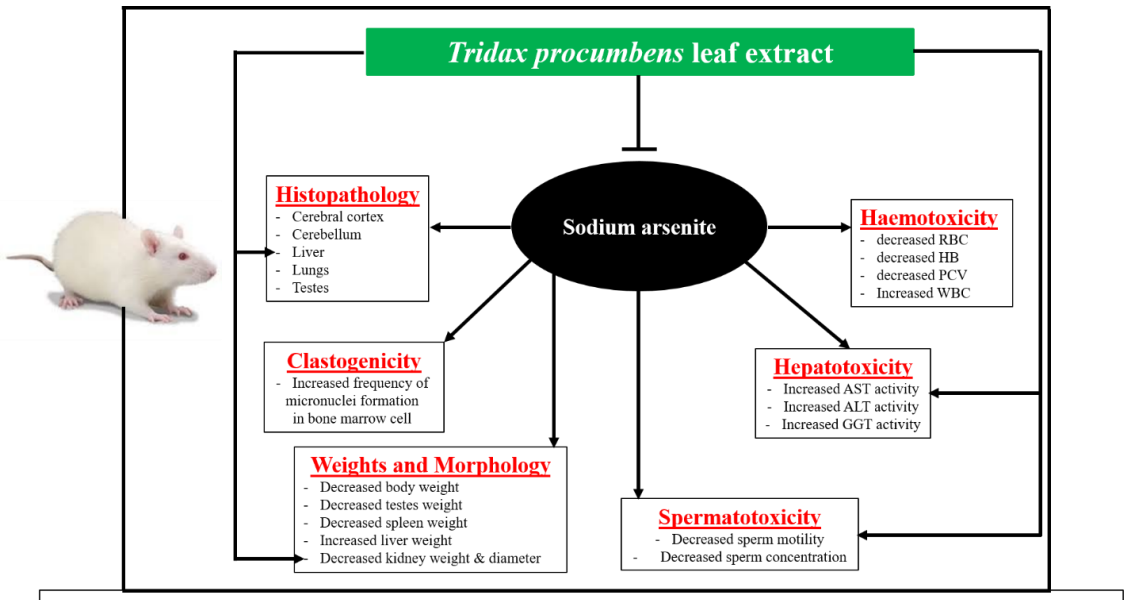
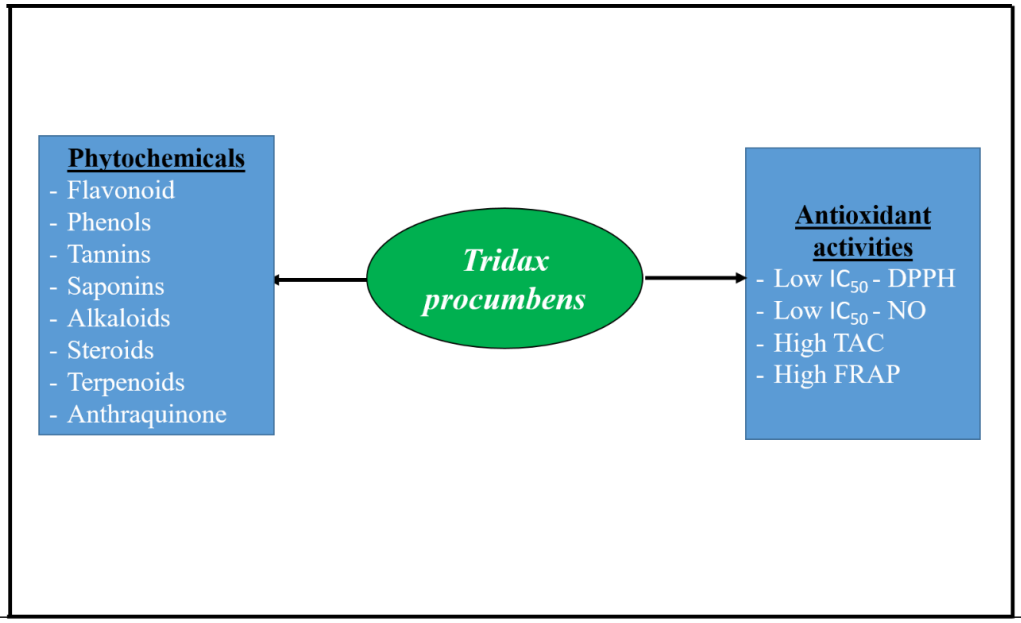
APPENDIX



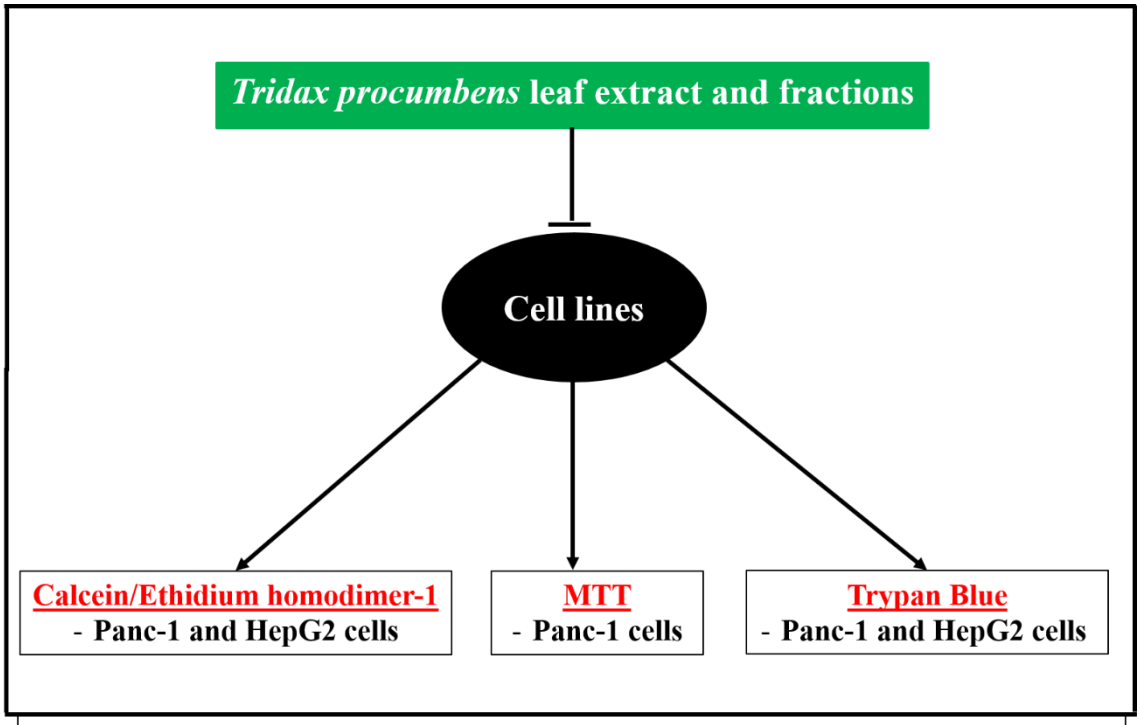
Schematic flow chart 2: *Tridax procumbens* leaf extract modulates dysfunctions mediated by sodium arsenite in male Wistar rats

Appendix I



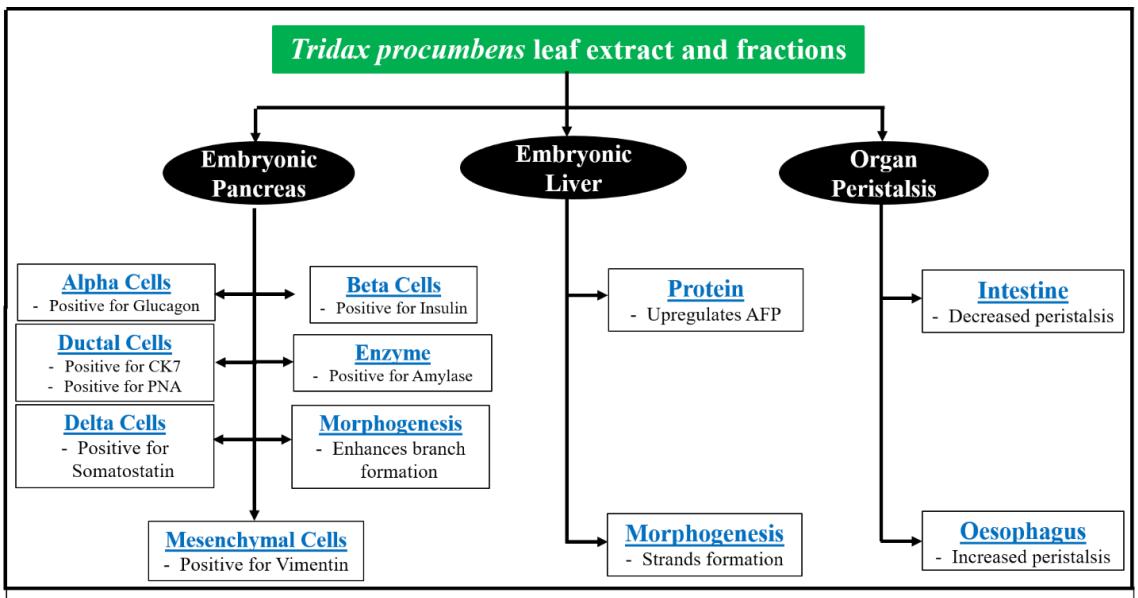
Schematic flow chart 3: Phytochemical constituents and antioxidant activities of *Tridax procumbens* leaf extract and fractions

Appendix II



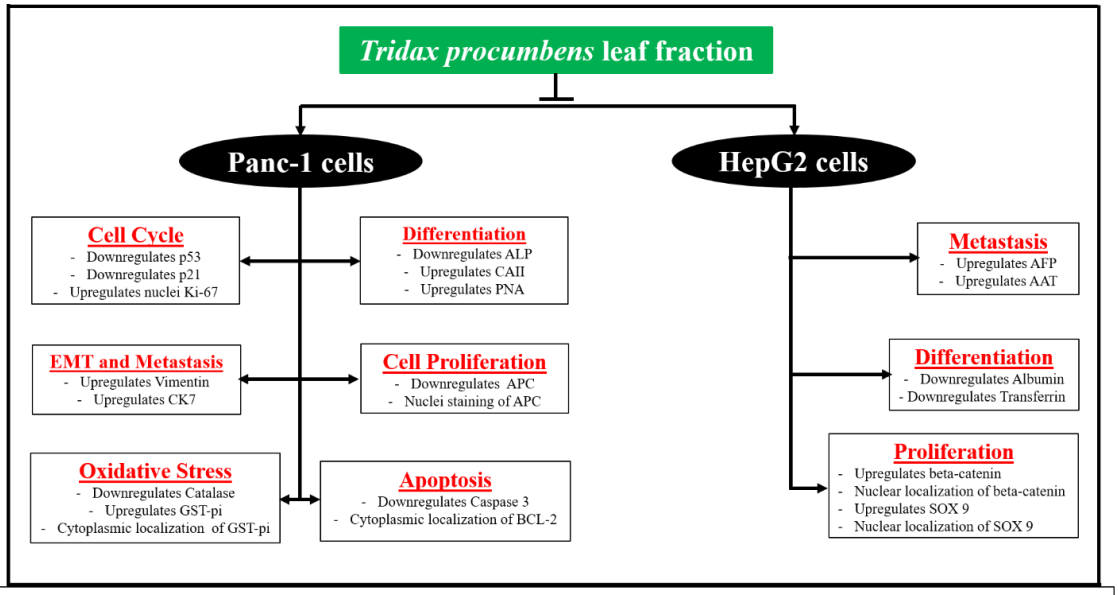
Schematic flow chart 4: *Tridax procumbens* leaf extract and fractions elicited death in Panc-1 and HepG2 cells in a time and dose dependent manner

Appendix III



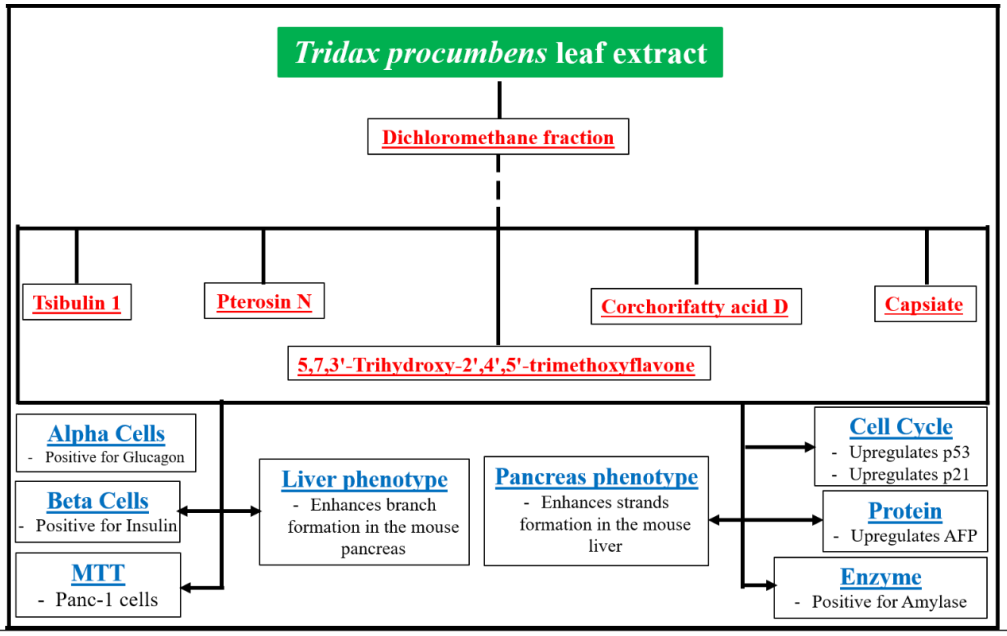
Schematic flow chart 5: ***Tridax procumbens*** **caused phenotype morphogenesis, modulates peristalsis and upregulates markers specific for embryonic mouse pancreas and liver**

Appendix IV



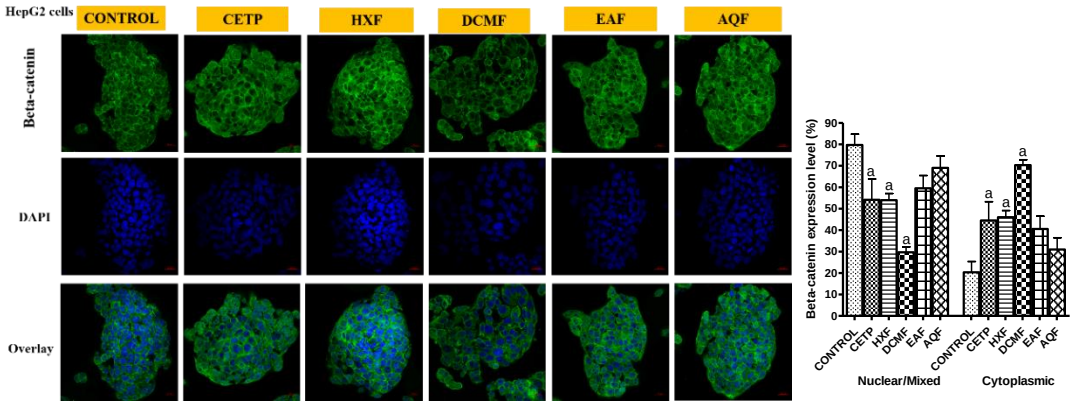
Schematic flow chart 6: *Tridax procumbens* fraction suppresses Panc-1 and HepG2 cells through markers of oxidative stress, apoptosis, proliferation, differentiation and metastasis

Appendix V

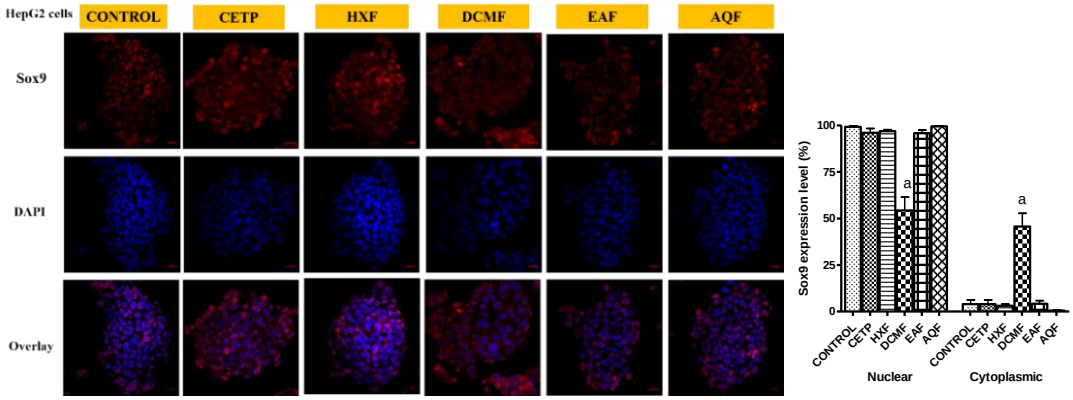


Schematic flow chart 7: The modulatory activities of *Tridax procumbens* fraction in embryonic mouse tissues and cancer cell lines were retained by isolated bioactive compounds

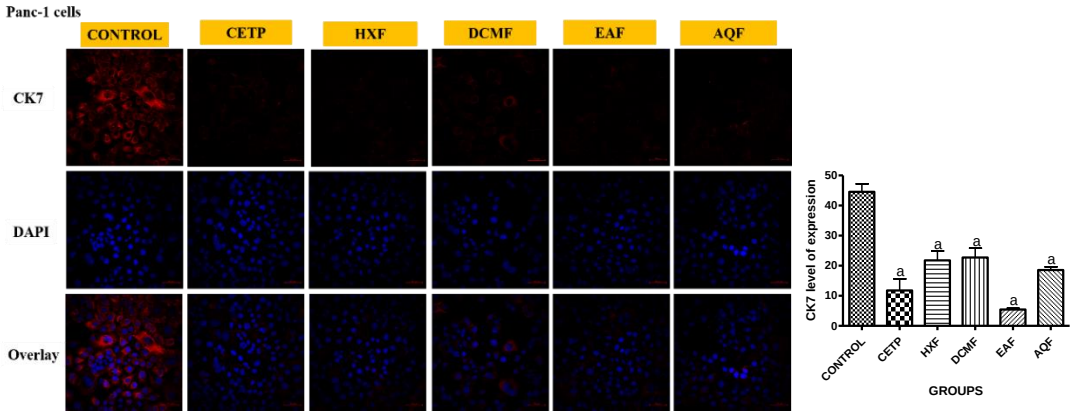
Appendix VI



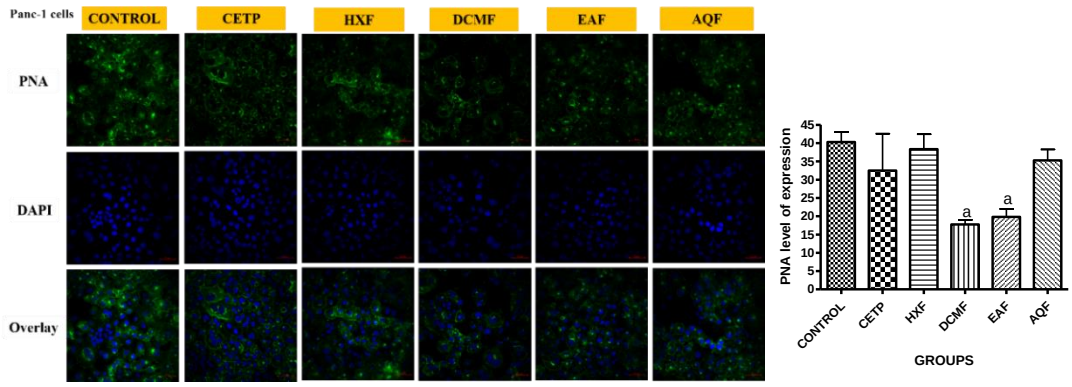
Appendix VII. The extract and fractions of *Tridax procumbens* downregulated beta-catenin after treatment (20 µg/ml) for 48 hours. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.



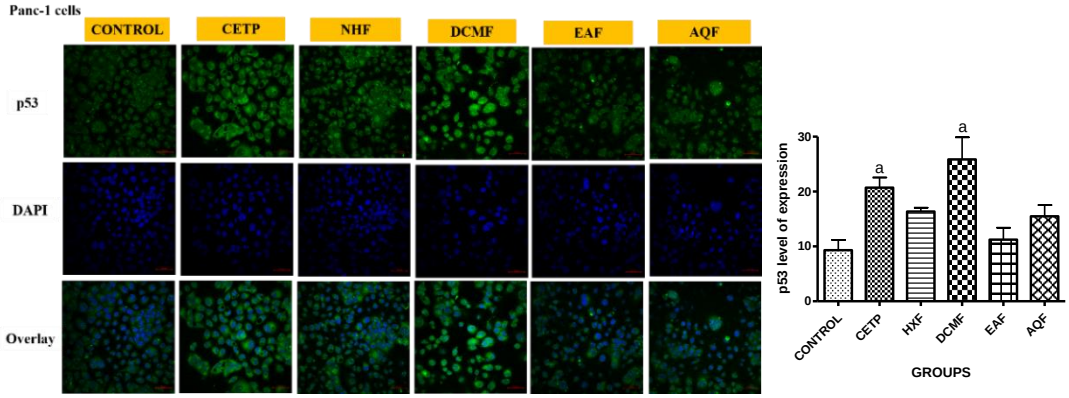
Appendix VIII. The extract and fractions of *Tridax procumbens* reduced Sox9 nuclear localisation after treatment (20 µg/ml) for 48 hours. DCMF produced significant cytoplasmic localisation of Sox9. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.



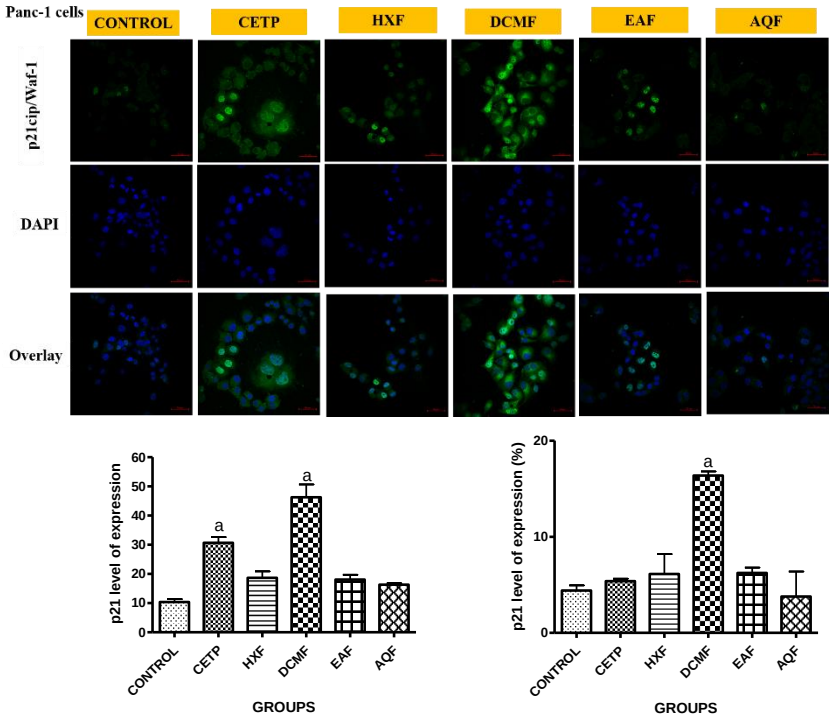
Appendix IX. The extract and fractions of *Tridax procumbens* downregulated cytokeratin 7 (CK7) after treatment (20 µg/ml) for 48 hours. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.



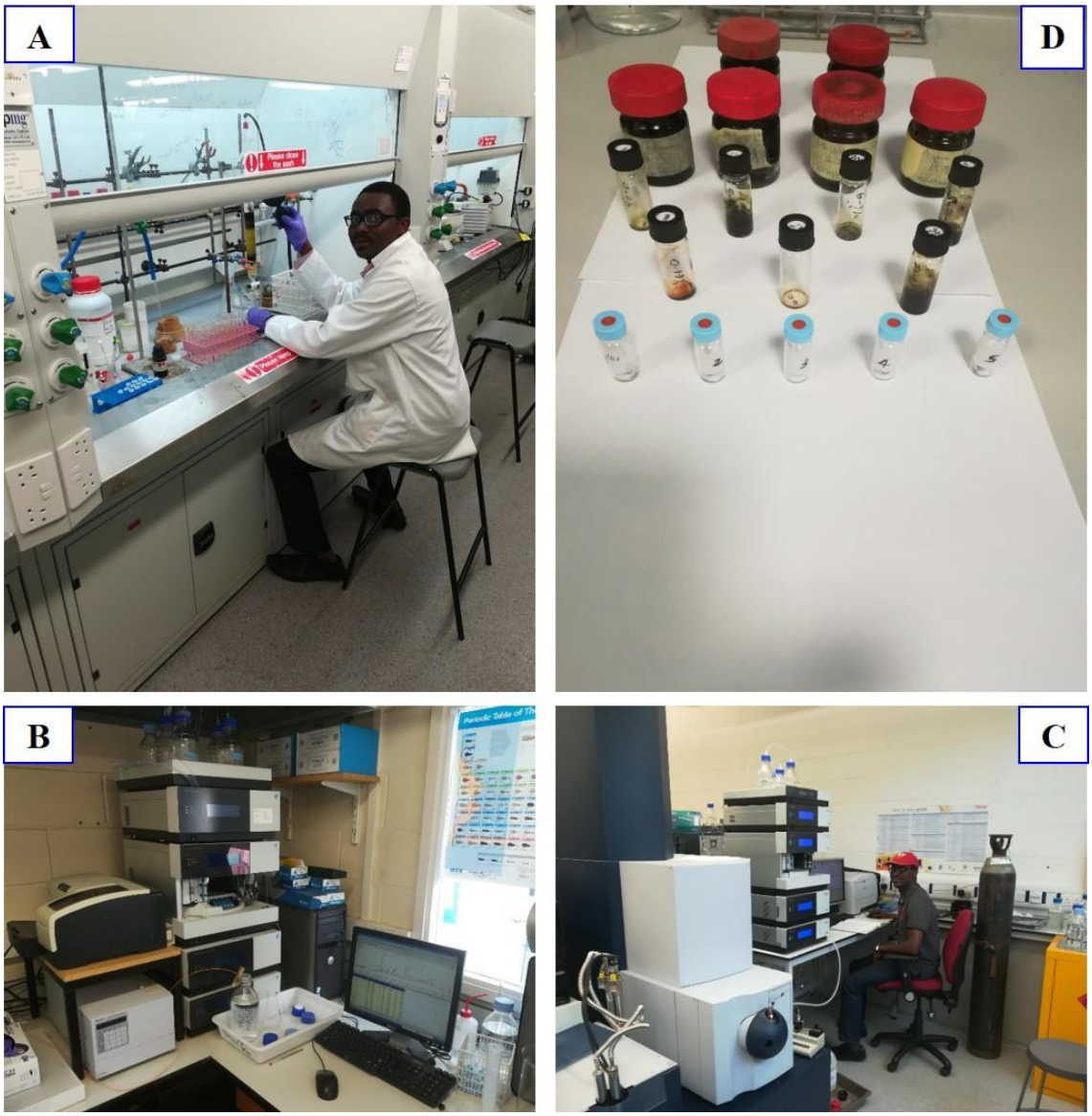
Appendix X. The extract and fractions of *Tridax procumbens* downregulated peanut agglutinin (PNA) after treatment (20 µg/ml) for 48 hours. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.



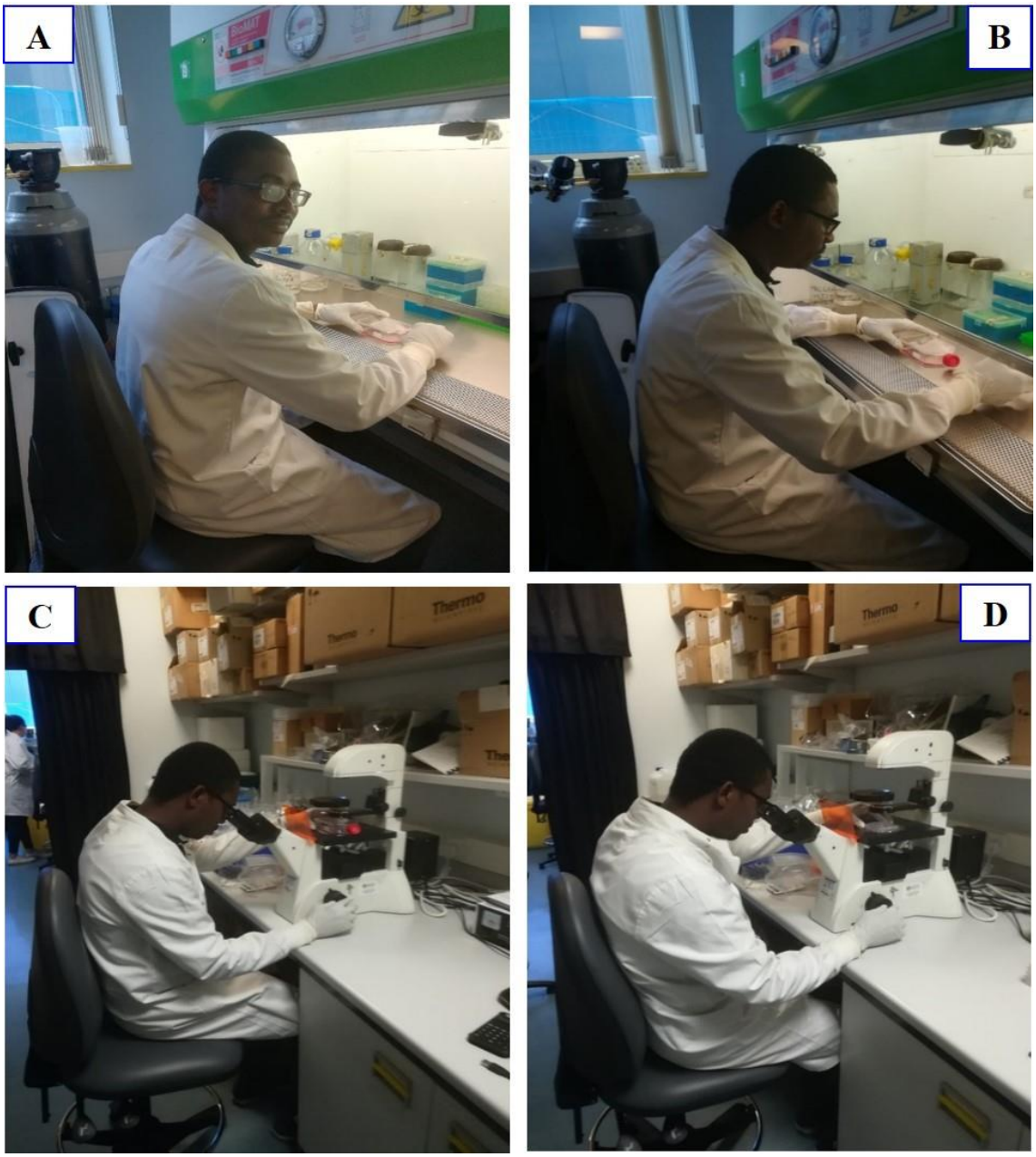
Appendix XI. The extract and fractions of *Tridax procumbens* upregulated p53 after treatment (20 µg/ml) for 48 hours. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.



Appendix XII. The extract and fractions of *Tridax procumbens* upregulated p21 after treatment (20 µg/ml) for 48 hours. [a]significant ($p < 0.05$) from control. Control (dimethyl sulfoxide), CETP (crude extract of *Tridax procumbens*), HXF, DCMF, EAF, AQF (hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively). n = 3.

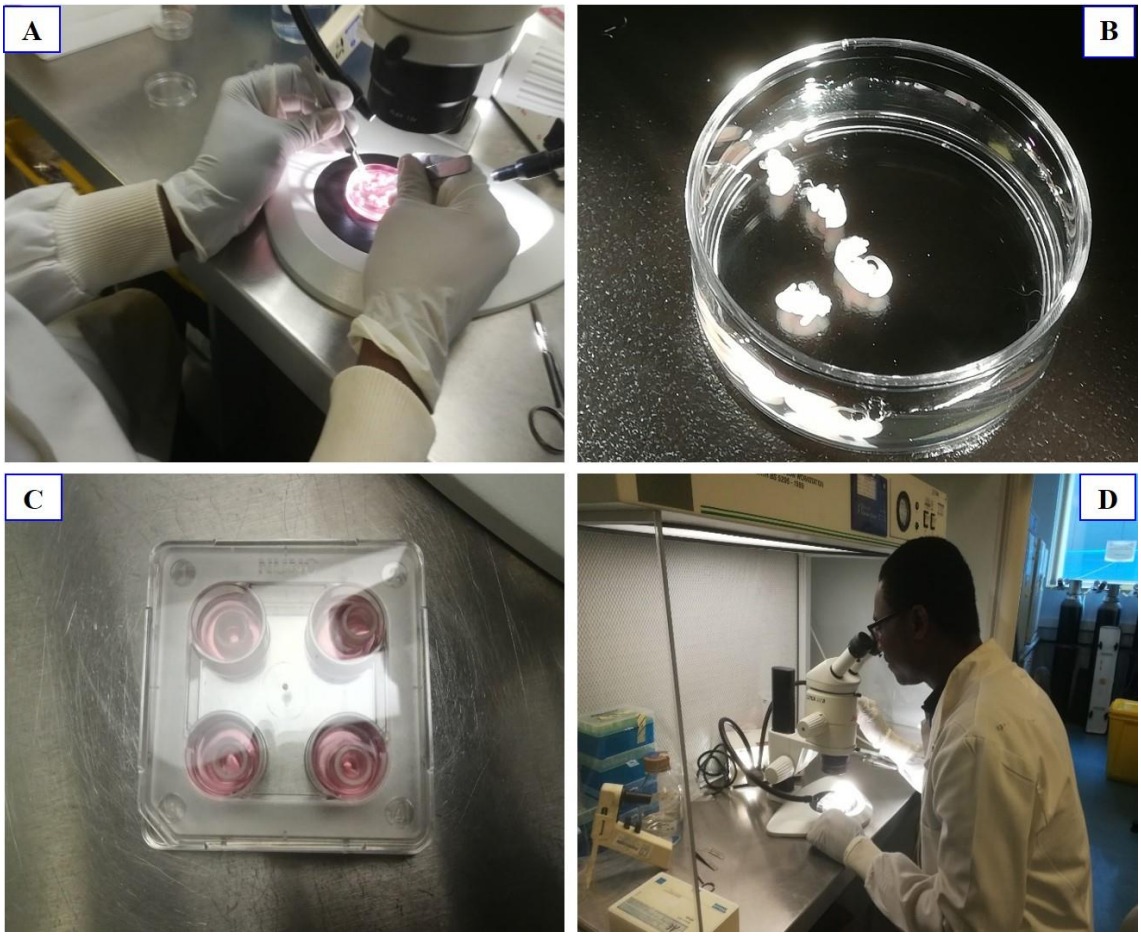


Appendix XIII. Identification of bioactive compounds with chromatographic and spectrometric techniques. A (column chromatography section), B (HPLC section), C (MS/MS section), D (test samples obtained after extraction).



Appendix XIV. Cell counting and passaging. A (preparation of cells), B (removal of medium), C (viewing cells for complete trypsinisation), D (viewing cells for proper seeding).

Appendix XV. Systematic dissection of E11.5d pregnant mouse using jeweller's forceps, sharp scissors. A (preparation of table for dissection), B (opening of pregnant mouse), C (removal of embryo from pregnant mouse), D (separation of embryo from the uterus).



Appendix XVI. Preparation of dissected embryonic mouse tissues for culturing using Jeweller's forceps, sharp scissors, fine tungsten needle and dissecting microscope (Leica m28, m275). A (decapitation of embryo and isolation of tissue of interest), B (decapitated embryo), C (isolated tissue of interest in cylinder cloning rings), D (use of dissecting microscope to assess proper positioning of tissue).

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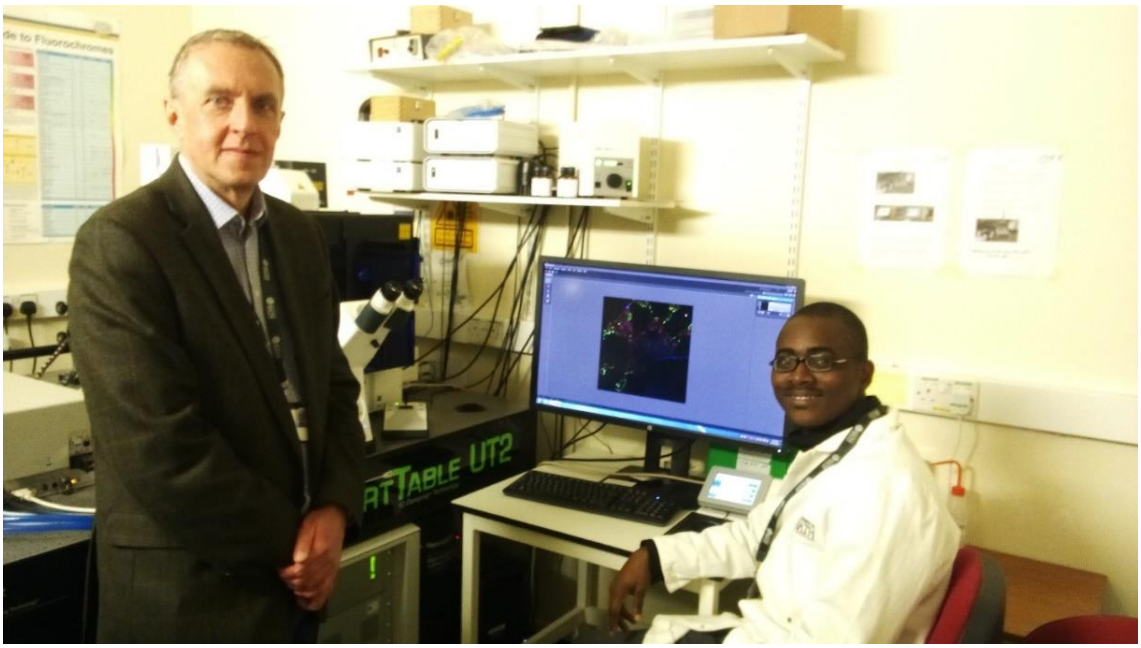
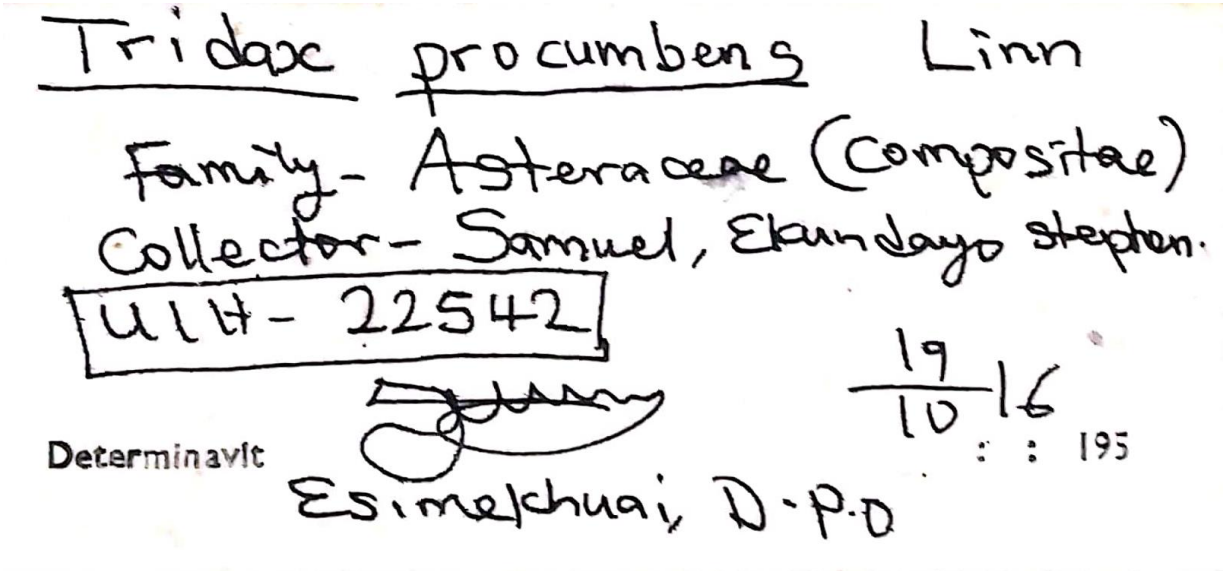
Appendix XVII. Meeting with supervisor, Professor Oyeronke A. Odunola, to discuss project results and the way forward.



Appendix XVIII. Collection of immunofluorescence images using high-throughput confocal microscope (LSM 880).



Appendix XIX

ANIMAL CARE USE AND RESEARCH ETHICS COMMITTEE (ACUREC)

UNIVERSITY OF IBADAN

08176917269

E.mail: animaluseresearch@gmail.com / animaluseresearch@yahoo.com

Our Ref: ..... Your Ref: .....

Date: .....

U.I. ACUREC/SES/29-12-2016

29th December, 2016

SAMUEL, Ekundayo Stephen,
Department of Biochemistry,
Faculty of Basic Medical Sciences,
University of Ibadan.
Ibadan.

NOTICE OF ETHICAL APPROVAL FOR A RESEARCH PROJECT PROPOSAL

On behalf of the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC), I write to grant you an ethical approval to carry out your research project work titled: **"THE EFFECT OF THE ETHANOL LEAF EXTRACT OF TRIDAX PROCUMBENS L. ON SODIUM ARSENITE-INDUCED TOXICITY IN MALE ALBINO (WISTAR STRAIN) RATS "**

refers: strictly as outlined in your proposal submitted for assessment.

Please quote **UI-ACUREC/App/12/2016/03** as reference for this approval.

You are to note that **UI-ACUREC** reserves the right to monitor and conduct compliance visit to your research site without previous notification.

Thank you.

Prof. S.I.B. Cadmus
Chairman UI-ACUREC

NB: The committee reserves the right to revoke this approval if there is non-compliance to the approved proposal concerning ACUREC guidelines.

Chairman: Professor S. I. B. Cadmus (DVM, Ph D)
Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Ibadan, Nigeria

Appendix XX

Animals (Scientific Procedures) Act, 1986
Universities' Training Group

No: 42787

This is to certify that **Ekundayo Samuel**

has successfully completed the following programme of training, as required under the UK and EU training framework and approved by the Universities' Accreditation Scheme at the University of Bath

Personal Licence Category B Training Course

UK Modules	*EU Modules**	*Species Specific*
L	(Module 1)	no
E1	(Module 2)	no
PIL A (theory)	(Modules 3.1, 4, 5, 7)	yes
PIL A (skills)	(Modules 3.2, 8)	yes
K (theory)	*(Module 6.1)*	*no*
K (skills)	(Module 6.2)	yes
PIL B	(Module 20)	no

Species: — Rat, Mouse, Guinea Pig —

These modules satisfy the training requirements for Personal Licence category B.

Signed: L. Taylor for Universities' Training Group
(L. Taylor - Secretary)

Date: 13 November 2017

In the UK, competence in procedures, including euthanasia, using living animals, which could cause pain, suffering, distress or lasting harm, will only be achieved, under supervision, when a person begins their work.

Please note, this Certificate is not a licence to perform regulated procedures under the Animals (Scientific Procedures) Act 1986

EU Modules are described in the common education and training framework to fulfil the requirements under Directive 2010/63/EU; http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_E-T.pdf

Appendix XXI

Appendix XXII: List of Publications and Abstracts in Journals and Conference Proceedings

1. **Samuel, E.S**., Tosh, D. and Odunola, O.A. 2022. A semi-purified fraction of *Tridax procumbens* induces differentiation of mouse embryonic pancreas and enhances the population of liver progenitor cells. *Proceedings of the ACS Spring 2022 National Meeting & Exposition*, San Diego, CA, USA, 20-24 March. *ACS Publication SciMeetings*.
2. **Samuel, E.S.**, Gbadegesin, M.A., Owumi, S.E., Aina, O.O., Olugbami, J.O., Adegoke, A.M. and Odunola. O.A. 2021. Leaf extract of *Tridax procumbens* remediates sodium arsenite-induced toxicity in male Wistar rats. *Proceedings of the 7th Unibadan Conference of Biomedical Research 2021*, University of Ibadan, Ibadan, Nigeria, 13-18 September.
3. **Samuel E.S.,** Tosh, D. and Odunola, O.A. 2020. Modulation of markers of cellular proliferation, growth and metastasis by *Tridax procumbens* in HepG2 cells. *Proceedings of the ACS fall 2020 National Meeting & Exposition*, San Francisco, CA, USA, 17-20 August. *ACS Publication SciMeetings* https://doi.org/10.1021/scimeetings.0c07372.
4. **Samuel, E.S.,** Tosh, D. and Odunola, O.A. 2020. *Tridax procumbens* suppresses cellular markers of tumour growth, proliferation and invasiveness in pancreatic ductal adenocarcinoma cell line. *The FASEB Journal* 34.1 supplement: 932.8-932.8. Doi.org/10.1096/fasebj.2020.34. s1.02083
5. **Samuel, E.S.,** Akinola, T.J., Tosh, D. and Odunola, O.A. 2019. The antioxidant and cytotoxic properties of *Tridax procumbens* leaf extract on liver and pancreatic cancer cell lines. *Proceedings of the Experimental Biology conference*, Orlando, Florida, USA, 21-25 April. *The FASEB Journal* 32.1 supplement: 932.8-932.8.
6. **Samuel, E.S.,** Olopade, J.O., Gbadegesin, M.A. and Odunola, O.A. 2018. Tissue morphology and expression of myelin basic protein and inducible nitric oxide synthase in Wistar rats treated with sodium arsenite: The beneficial effects of ethanol leaf extract of *Tridax procumbens*. *Proceedings of the Experimental Biology conference*, San Diego CA, USA, 21-25 April. *The FASEB Journal* 32.1 supplement: 932.8-932.8.

7. **Samuel, E.,** Gbadegesin, M, Tosh, D. and Odunola, O. 2018. Treatment of albino Wistar rats with the leaf extract of *Tridax procumbens* Linns reduces sperm motility and the number of sperm. *Department of Biology and Biochemistry Research Day 2018*, Department of Biology and Biochemistry, University of Bath, Bath, UK, 1 February.
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