

A mechanistic study of organochlorine hepatotoxicity

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Declaration

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Abstract

Pentachlorophenol, (PCP) is an organochlorine compound which was first developed in the 1930's. PCP is said to be the most toxic of the chlorophenols and is classified as a hazardous substance and a probable human carcinogen. PCP has proven to be cytotoxic to a number of cell lines translating to its effect on various organs.

The aim of the study was to assess organochlorine-induced hepatotoxicity in a mechanistic manner using an in-house developed procedure. Also, the possible hepatoprotective effect of methanolic extracts of the bark of two medicinal plants, *Burkea africana* (BA) and *Syzygium cordatum* (SC), as well as the known hepatoprotective agent, N-acetyl cysteine (NAC), were investigated.

In addition to PCP, two of its major metabolites, tetrachloro-1,2-hydroquinone (TCHQ) and tetrachloro-1,4-benzoquinone (TCBQ) were also evaluated. A hepatocarcinoma cell line (HepG2) was used to investigate the effect of these compounds on different parameters of cellular function. Cytotoxicity was assessed using the neutral red uptake assay. Cytochrome P4501A1 (CYP1A1) activity was determined using ethoxy-resorufin-*O*-deethylation as surrogate. Generation of reactive oxygen species (ROS) was investigated by measuring dichlorofluorescein diacetate cleavage. Effects on mitochondrial membrane potential were determined using JC-1 staining, whilst necrosis was investigated by assessing plasma membrane integrity using propidium iodide (PI) staining. The degree of apoptotic death was determined by quantifying caspase-3 activity. Assays were repeated with an additional 1 h pre-treatment of the cells with either NAC, SC or BA in order to investigate whether these compounds were able to protect against the toxicity induced by PCP and its metabolites.

The IC₅₀ values of PCP, TCHQ and TCBQ were 68.0, 144.0 and 129.4 μ M, respectively. All three test compounds induced CYP1A1 activity with PCP being the most potent. TCBQ produced extensive ROS generation. TCHQ also induced ROS

generation, whilst PCP appeared to have no significant effect on ROS generation. All test compounds caused mitochondrial depolarization. None of the test compounds caused an increase in necrotic cell death. PCP, TCHQ and TCBQ had negligible effects on apoptosis.

Both SC and BA alleviated the toxic effects observed in cells treated with PCP. Minor increases in viability occurred in cells pre-treated with plant extracts prior to exposure to both metabolites. NAC, as well as both plant extracts, greatly reduced CYP 1A1 activity induced by PCP. NAC, SC and BA exacerbated CYP1A1 induction in cells exposed to concentrations of TCBQ and TCHQ that initially produced little or no effect on CYP1A1 activity. Contrarily, decreased CYP1A1 activity was observed in cells exposed to concentrations of TCBQ and TCHQ where extensive induction of CYP1A1 occurred. NAC, as well as both plant extracts, suppressed ROS generation in cells exposed to all test compounds. In cells exposed to PCP and TCBQ more extensive mitochondrial depolarization was seen when pre-treated with NAC and plant extracts than when exposed to the compounds alone. Negligible effects were seen in pre-treated cells exposed to TCHQ. BA and SC caused increases in necrotic death in cells exposed to the test compounds. NAC, BA and SC had negligible effects on the changes in caspase-3 activity induced by the test compounds.

From the results it is proposed that PCP induces its own metabolism by increasing CYP1A1 activity. It also causes mitochondrial insult which could lead to the opening of the mitochondrial permeability transition pore and subsequent release of cytochrome C, activation of caspases and eventually apoptotic cell death. With regard to TCHQ and TCBQ, results suggest that extensive ROS generation caused damage to various cellular macromolecules and that this could be the main cause of their toxicity.

NAC, SC and BA appeared to alleviate toxicity in certain instances. Further investigation is required in order to assess them as possible hepatoprotective agents.

Keywords: *Burkea africana*, Hepatotoxicity, *In vitro*, N-acetyl cysteine, Organochlorine, Pentachlorophenol (PCP), *Syzygium cordatum*, Tetrachloro-1,4-benzoquinone (TCBQ), Tetrachloro-1,2-hydroquinone (TCHQ),



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List of Abbreviations

AIF	Apoptosis inducing factor
AAPH	2,2'-azobis-2-methyl-propanimidamidedihydrochloride
ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
AMC	7-amino-4-methylcoumarin
APAF-1	Apoptotic protease activation factor
ARNT	Aryl hydrocarbon nuclear translocator
ATP	Adenosine triphosphate
BA	<i>Burkea africana</i>
bHLH	Basic helix-loop-helix
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central nervous system
DCFDA	Dichlorofluorescein diacetate
DCFH	Dichlorofluorescein
DDT	Dichlorodiphenyltrichloroethane
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid

EC ₅₀	Concentration that is effective in 50% of the population
EDTA	Ethylenediamine-tetraacetic acid
EndoG	Endonuclease G
EMEM	Eagle's minimum essential medium
ER	Endoplasmic reticulum
EROD	Ethoxy-resorufin- <i>O</i> -deethylation
FADD	Fas-associated death domain
FADH ₂	Flavin Adenine Dinucleotide (reduced form)
FasL	Fas Ligand
GSH	Glutathione
GST	glutathione- <i>S</i> -transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heat shock protein
IC ₅₀	Concentration that is inhibitory in 50% of the population
JC-1	5,5,8,6,6,8-tetrachloro- 1,18,3,38-tetraethylbenzimidazolyl-carbocyanine iodide
LC ₅₀	Concentration that is lethal in 50% of the population
LDH	Lactate dehydrogenase
mm	Millimetre
MMP	Mitochondrial membrane potential
MPT	Mitochondrial permeability transition

mRNA	Messenger ribose nucleic acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide
NAC	<i>N</i> -Acetyl cysteine
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
NAPQI	N-acetyl-p-benzoquinone imine
NK	Natural Killer
NRU	Neutral Red uptake
p53	protein 53
PBS	Phosphate buffered saline
PCP	Pentachlorophenol
PI	Propidium iodide
pH	A measure of the acidity or the alkalinity of a solution as measured on a scale of 0 to 14
PMSF	Phenylmethanesulfonyl fluoride
PNS	Peripheral nervous system
ROS	Reactive oxygen species
SMAC/DIABLO	Second mitochondria-derived activator of caspase/direct IAP binding protein with low Pi
SC	<i>Syzygium cordatum</i>
TCBQ	Tetrachloro-1,4-benzoquinone
TCHQ	Tetrachloro-1,2-hydroquinone

TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-associated death domain
WHO	World Health Organization
XRE	Xenobiotic response element

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Chapter 1 – Literature Review

1.1 Organochlorine Pesticides

Organochlorine insecticides were first produced after World War II. Their low cost and efficacy led to their widespread use in agriculture as insecticides and in public health to control vectors of disease¹.

Their chemical composition consists of carbon, chlorine, hydrogen, and in some instances oxygen atoms as well as carbon-chlorine bonds and cyclic carbon chains such as benzene rings¹. Due to their chemical stability they are resistant to degradation and persistent in the environment^{2,3}. As they are lipophilic substances, they accumulate in the fat of an organism, increasing in concentration at each trophic level resulting in bioaccumulation and biomagnification^{2,4}.

In the 1960's the harmful effects of organochlorine pesticides were established and their use was restricted and banned in many countries. However, they are still used in some developing countries today and, due to their resistance to degradation, are still ubiquitous in the environment in many areas of the world¹. Many organochlorine insecticides have been shown to be endocrine disrupters and enzyme inducers. These compounds are said to be responsible for a large variety of adverse effects including effects on the immune system, neurotoxic effects and impairment of thyroid function^{2,5}. They have also been linked to certain types of cancers such as breast cancer, non-hodgkins lymphoma, leukemia, uterine cancer and liver cancer^{1,4}.

Organochlorine pesticides can be characterised into sub groups according to their chemical structure⁶. These sub groups include benzene hexachloro isomers such as lindane, cyclodienes such as dieldrin, aldrin, endrin, heptachlor, endosulphan and chlordane, as well as dichlorodiphenyltrichloroethane (DDT) and analogues such as methoxichlor, chlorobenzylate and dicofol⁶.

The sub groups have various effects on the liver. Lindane is a broad spectrum insecticide and is classified by the World Health Organisation (WHO) as moderately

hazardous⁶. It alters cellular anti-oxidant homeostasis, resulting in hepatic oxidative stress which is considered to be the main mechanism of its toxicity^{7,8}. It results in the induction of liver cytochrome P450 enzymes (CYPs) and increased superoxide radical formation⁹. Lindane has been found to induce hepatocarcinomas in both mice and rats¹⁰. Dieldrin is a versatile soil insecticide classified by the WHO as highly hazardous⁶. Dieldrin toxicity has led to the formation of hepatocarcinomas in mice but not rats¹⁰. It has been suggested that its carcinogenic effect in mice could be attributed to a species selective induction of reactive oxygen species (ROS)¹⁰. A major target of dieldrin toxicity is mitochondria. It inhibits mitochondrial phosphorylation and thus reduces adenosine triphosphate (ATP) production¹¹. It has also been reported to activate caspase-3 in a hepatocarcinoma cell line¹¹. DDT is a non-systemic insecticide classified by the WHO as moderately hazardous⁶. Induction of liver microsomal enzymes has been reported to be one of the earliest effects of DDT toxicity⁶. DDT has been reported to cause hepatocellular necrosis *in vivo*¹². Its carcinogenic effect has also been studied extensively in animal models¹².

Although the use of organochlorine pesticides has been banned or severely restricted in most first world countries, it is evident that their hepatotoxic effect still has and will remain to have serious implications for mankind in the future.

1.1.1 Pentachlorophenol

Pentachlorophenol (C₆HCl₅O), (PCP), is an organochlorine¹³ compound which was first developed in the 1930's¹⁴. The structure of PCP is depicted in Figure 1. At room temperature, PCP is a colourless crystalline substance¹⁵. At high temperatures PCP has a distinct phenolic smell¹⁵. In the past, PCP was extensively used as a wood preservative^{13,14,16-20}, herbicide^{13,17,19,21}, insecticide^{13,21}, defoliant^{13,17}, fungicide^{13,21-23}, molluscicide^{13,21}, algicide^{13,21}, disinfectant^{20,21}, germicide¹³ and as an ingredient in

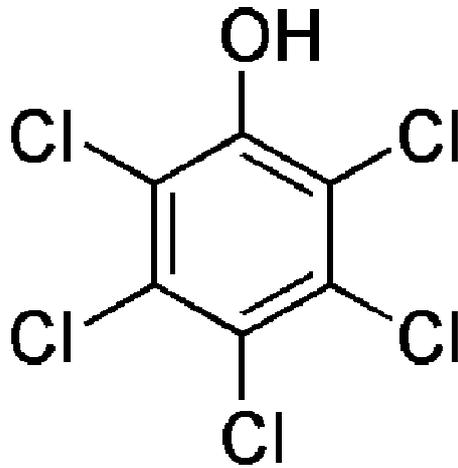


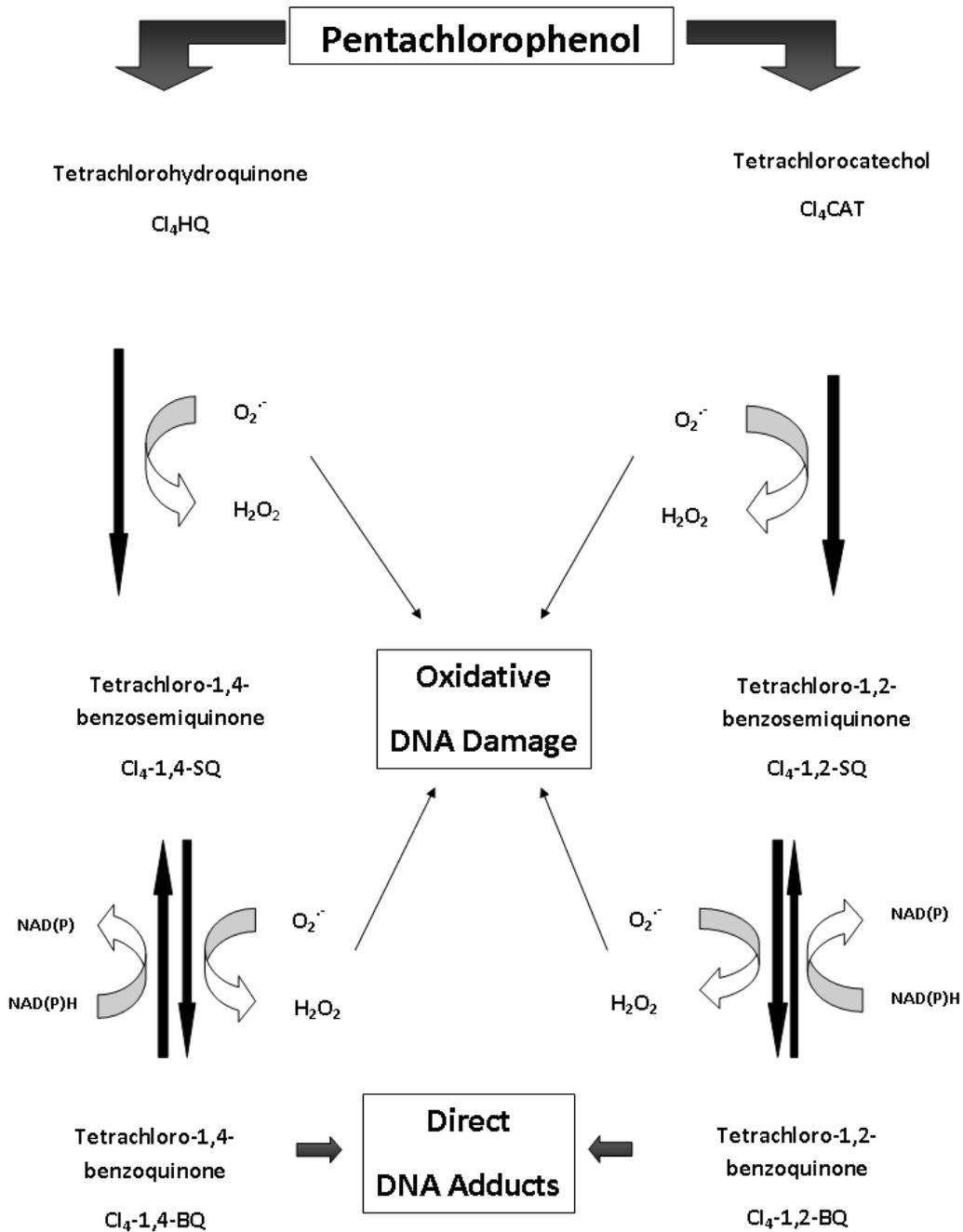
Figure 1. Chemical structure of PCP.

anti-fouling paints²¹. Its numerous uses in the past, coupled with its stability, makes PCP a persistent organic pollutant.

In humans PCP can enter the body via dermal absorption, inhalation and ingestion of contaminated food substances^{13,24,25}.

To eliminate PCP from the body, it is first metabolized to its quinols; tetrachloro-1,2-hydroquinone (TCHQ) and tetrachlorocatechol. TCHQ and tetrachlorocatechol are then oxidized to their corresponding semi-quinones, tetrachloro-1,4-benzosemiquinone and tetrachloro-1,2-benzosemiquinone, respectively. The semi-quinones can then be further oxidized to their quinones, tetrachloro-1,4-benzoquinone (TCBQ) and tetrachloro-1,2-benzoquinone, respectively^{19,26,27}. Figure 2 illustrates the metabolism of PCP.

PCP is said to be the most toxic of the chlorophenols¹⁴ and is classified as a hazardous substance¹⁵ and a probable human carcinogen¹⁵. Symptoms of exposure to PCP may



(Adapted from Lin et al.¹⁷)

Figure 2 Metabolism of PCP¹⁷.

include fatigue, lack of concentration, sleeplessness, headache, irritation of mucous membranes and nausea²⁸. Acute exposure to high quantities of PCP may be fatal and is associated with extreme weakness, hyperpyrexia and profuse sweating. These effects can be explained by the uncoupling of oxidative phosphorylation in the mitochondria by PCP²⁵.

PCP has proven to have toxic effects on various cell lines. Among these are a number of hepatic cell lines. Some of the cellular effects that PCP has on these cells are illustrated in Table 1.

PCP has shown *in vivo* toxicity in various species, some of which include rodents^{19,27,29,30}, birds^{15,29}, rabbits¹⁵ as well as fish^{15,20} and other aquatic species^{15,31}. *In vivo*, PCP exerts its effect on various organs including the liver (adverse effects on metabolism)^{13,15}, skin, kidney, gastro-intestinal tract, central nervous system (CNS) and peripheral (PNS)¹⁵. Other effects include endocrine–disrupting functions and immunotoxic effects^{13,15}. Endocrine effects include reduction of blood levels of thyroid hormones^{15,32,33}, anti-estrogenic activity as well as anti-androgenic activity^{13,34,35}. Reduction of thyroid hormone levels can lead to detrimental metabolic and growth effects whilst changes in sex hormones may adversely affect reproduction. PCP exerts its immunotoxic effect by decreasing natural killer (NK) cell function as well as other mononuclear cells (such as T-cell) function^{18,21}, decreasing IgM antibody response^{13,15} as well as inhibiting the complement system¹⁵.

Cancer concerns regarding PCP first arose in the 1970's²². Since then it has been adequately shown in animal models that there is an existing relationship between PCP and cancer^{22,34}. Types of cancers observed in animals exposed to PCP include hepatocellular adenomas, adrenal medullary pheochromocytomas, hemangiosarcomas, malignant mesothelioma, nasal squamous cell carcinoma, lymphomas and hepatocellular carcinoma^{13,30,36}. Cancers that have been found in humans after PCP exposure include non-Hodgkin's lymphoma, soft tissue sarcoma, multiple myeloma, lung, kidney, nasopharyngeal and sinus cancers^{22,36}.

Table 1. Cellular effects of PCP on hepatocytes.

Compound	Concentration	Cell line	Exposure time	Parameter	Effect	Reference
PCP	20- 500µM	Chang human liver cell	24 and 48 h	Cell viability	Decrease	Wang et al. ³⁷
	25 – 50 µM		> 24 h	Apoptosis	No effect	
PCP	250µM	HepG2	24 h 48 h	Cell viability	Decrease	Wang et al. ³⁸
PCP	Serial doses 0 - 100µg/ml (0 – 375.46 µM)	HepG2	48 h	Cell viability	Decrease	Dorsey et al. ³⁹
				CYP1A1	Increase	
				XRE	Increase	
				HMTIIA	Increase	
				C-fos	Increase	
				GADD153	Increase	
PCP	0 to 31.0 µg/mL (0 – 116.39 µM)	AML 12 mouse hepatocytes	48h	Cytotoxicity	Decrease	Dorsey ⁴⁰
	0, .484, .968, 1.94, 3.87, 7.75, 15.5, and 31 µg/mL (1.8µM - 116.39 µM)			Mitogenic effect	Increase	Jiang et al. ⁴¹
PCP	> 7.75 µg/mL (> 29.10 µM)	AML 12 mouse	48h	Cell viability	Decrease	Dorsey ⁴²



		hepatocytes				
	0-22 µg/mL (0-82.60 µM)			Caspase-3 activity	Increase	
				Oxidative stress	Increase	
				c-fos	Increase	
				GADD 153	Increase	
				HSP70	Increase	
				P53	Increase	
				Cyclin D1	Increase	
PCP	0.01, 0.1 10 and 100 µM	Primary carassius carrassius hepatocytes	8 h	Cell viability	Decrease	Dong et al. ¹³
				Effects on Ca ²⁺ ,Mg ²⁺ -ATPase activity	Decrease	
				[Ca ²⁺] _i	Increase	
				Effects on intracellular ROS, GSH and MDA	Increase	
				GSH	Decrease	
				MDA	Increase	



				DNA laddering	Increase at concentration > 10 μ M	
				Apoptosis	Increase	
				Caspase-3 activity	Increase at concentration > 10 μ M	
				ATP	Decrease at concentration > 1 Increase at concentration > 1 μ M	
				MMP ($\Delta\Psi$ m)	Decrease	
PCP	2.0, 4.0, and 6.0mg/L (7.51, 1.50, 22.53 μ M)	Primary Hepatocytes of Common carp	24h and 72h	CYP1A1 activity	Increase	Han et al. ⁴³
				GSH	Decrease	
				MDA	Increase	
	0.1 and 1.0 mg/L (0.38 and 3.80 μ M)			MMP ($\Delta\Psi$ m)	Slight increase	
				MMP ($\Delta\Psi$ m)	Decrease	
	2.0, 4.0, and 6.0mg/L (7.51, 1.50, 22.53 μ M)			Serum testosterone	Decrease	
				Plasma Vitellogenin	Decrease	

1.1.2 Tetrachlorohydroquinone and Tetrachlorobenzoquinone

TCHQ is the major metabolite of PCP³². It is a reactive quinone and is said to be more toxic than PCP^{32,44}. The structure of TCHQ is depicted in Figure 3.

TCHQ has been shown to exert cytotoxic effects on a number of cell lines such as: v79 cell⁴⁵ chinese hamster ovary cells⁴⁴, human fibroblast cells³², B6C3F1 mouse cells⁴⁵, human hepatocarcinoma cells (HepG2)⁴⁶.

TCHQ is able to bind DNA causing single strand breaks and mutations *in vitro*^{32,44,47,48}. It reduces colony-forming ability in human fibroblasts⁴⁷. TCHQ has also been reported to induce ROS generation, micronuclei formation and mutations *in vitro*^{27,32}. Furthermore, it causes p53 protein accumulation, glutathione depletion and carcinogenic effects in mice⁴⁹.

TCBQ, a metabolite of TCHQ, is a potent inhibitor of human glutathione-S-transferase (GST) isoenzymes⁵⁰. It is reduced by nicotinamide adenine dinucleotide (NAD) and reacts with dioxygen to form superoxide radical^{51,52}. The structure of TCBQ is depicted in Figure 3.

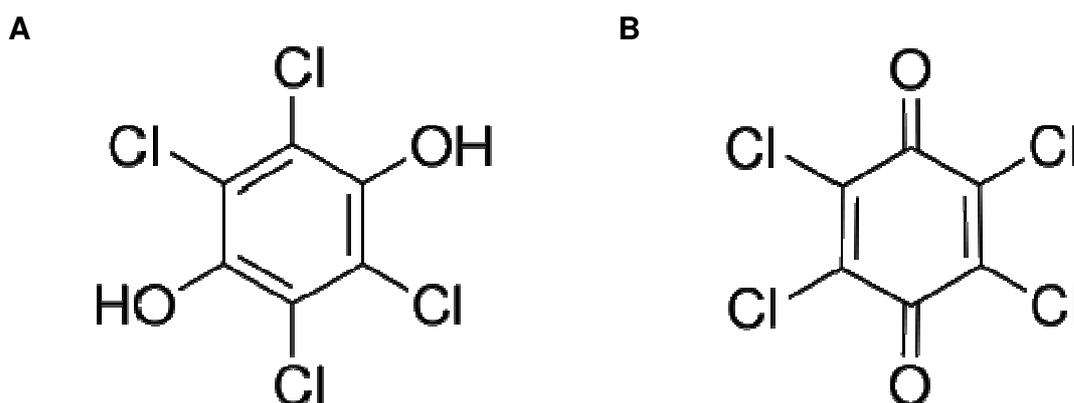


Figure 3. Chemical structures of A) TCHQ and B) TCBQ.

1.1.3 Relevance of Pentachlorophenol as test substance

Globally PCP's use has been restricted and its current indication is only as wood preservative in the timber industry. Even though its use has been restricted in most countries, its extensive use in the past and also its long persistence and its resistance to degradation has resulted in significant environmental contamination¹⁷. PCP is thus ubiquitous in air, soil and surface as well as ground water^{13,15,21}.

South African legislation to ensure the safe use of pesticides is poor⁵³. Third world countries have the inclination to use older, non-patented, less expensive but more acutely toxic pesticides which are environmentally persistent⁵⁴. Many pesticides which have been banned or restricted in first world countries continue to be registered for agricultural use in South Africa⁵³. Pentachlorophenol is one of these pesticides. Even though PCP has been restricted worldwide, it's freely available remains on the international market⁵⁴. Expenditures on pesticides in South Africa has increased from 325 million rands in 1985 to over 1,400 million rands by 1998⁵³. Pesticide exposure thus remains a hazard to farm workers⁵³. It is said that most of the data of acute pesticide poisonings come from the developing world⁵⁴. This can be attributed to the fact that most farm workers are uneducated and safe use of pesticides are not implemented⁵³.

There exists no specific antidote for treatment of PCP poisoning²⁴. Therefore treatment is only intensively supportive. Supportive treatment includes oxygen administration to prevent anoxia, fluid replacement, stabilizing electrolytes and most importantly fever control⁵⁵. It is recommended that fever should be reduced by physical means (sponge baths, fans) most antipyretics are thought to be of no use due to the peripherally-mediated mechanism of PCP induced hyperthermia and salicylate antipyretics are strongly contra-indicated as they also uncouple oxidative phosphorylation^{13,55}.

The fact that this highly toxic substance is still ubiquitous in the environment and that there exists no specific antidote provides rationale to investigate the full extent of the hepatotoxicity induced by PCP and its metabolites, it also provides motivation for testing substances for possible hepatoprotective effects against these substances.

1.2 Hepatotoxicity

Due to its unique metabolism and relationship to the gastrointestinal tract, the liver plays a central role in the functioning and homeostasis of an organism^{56,57}. It is key in the biotransformation of exogenous substances and is responsible for processing both dietary and xenobiotic components. The liver is involved in both phase I and phase II drug metabolism^{56,58}. Although the biotransforming capability of the liver allows for elimination of most toxic compounds it makes it an important target for xenobiotic toxicity^{57,59,60}.

Hepatotoxins can be classified as intrinsic or idiosyncratic hepatotoxins and the harmful effects caused by these toxins are usually defined as cytotoxic, genotoxic or metabolic^{60,61}. Cytotoxic effects are morphological and functional changes which result in the cellular death⁶⁰. Genotoxic effects refer to alterations and damage to cellular DNA and may result in tumour formation, whilst metabolic effects refer to direct effects on cellular metabolism which in turn effects cell function⁶⁰. A number of mechanisms can lead to the occurrence of these effects⁵⁷. It can occur due to the hepatotoxin itself or by its metabolite/s generated through CYPs^{57,62}. Phase I and II enzymes can be induced or inhibited by various hepatotoxins. Hepatotoxins are also able to impair ion transport or affect metabolic pathways by competing with normal cellular metabolites, resulting in cellular dysfunction or death⁶⁰.

The liver is the primary site for xenobiotic metabolism and foreign compounds entering the body usually find their fate in it^{63,64}. Even though xenobiotic metabolism usually allows for elimination of most toxic compounds, it is capable of modifying the pharmacological properties of substances activating inert chemicals into biologically reactive species^{57,60,63,64}. Metabolism of xenobiotics in hepatocytes can be divided into 3 phases: phase I metabolism, phase II metabolism and phase III metabolism. Phase I and II metabolism are responsible for converting lipophilic compounds to more hydrophilic compounds that can be excreted by the kidneys⁶⁵. Phase I metabolism involves oxidation, reduction and hydration reactions which commonly introduce nucleophilic groups into the substrate molecule⁶⁵. Phase II metabolism involves the conjugation of nucleophilic groups of xenobiotics or their metabolites with ionized

hydrophilic cofactors⁶⁵. Phase III metabolism is responsible for the transportation of xenobiotics and their oxidized metabolites in and out of hepatocytes⁶⁵. There are many factors which influence xenobiotic metabolism, these may include: age, sex, diet and alteration of hormonal status for example pregnancy and inhibition or induction of enzyme activity by certain environmental and pharmacological agents⁶⁴.

Induction of certain CYPs by hepatotoxins has been shown to result in hepatic injury^{66,67}. CYPs can be defined as a super family of heme proteins, which play a critical role in the metabolism of both endogenous and exogenous substances⁶⁸. A number of CYP sub families are inducible by various xenobiotics⁶⁹. *In vivo* and *in vitro* studies have allowed for the categorization of inducers into several classes on the basis of the spectrum of CYPs they induce and the potency of the induction⁶⁹ (Table 2). Certain substrates are capable of inducing their own metabolism and form biologically active metabolites⁶⁹. CYP1A1, encoded by *cyp1A1*, is particularly known for its inducibility^{68,70}. It oxygenates lipophilic substrates such as polycyclic aromatic hydrocarbons into water soluble metabolites that can eventually be excreted⁷⁰.

Table 2. Inducers of various CYPs.

Inducer	Enzyme
Polycyclic and polyhalogenated hydrocarbons	CYP1A1, CYP1A2
Phenobarbital-type chemicals	CYP2B
Ethanol type-molecules	CYP2E
Dexamethasone- or rifampicin-types	CYP3A
Clofibrate- or phthalate-types	CYP4A

A common mechanism of hepatotoxicity is mitochondrial dysfunction^{62,71}. Due to the central role of mitochondria in both energetic metabolism and thus cellular homeostasis, they are a target for toxic substances. Mitochondrial dysfunction may be caused by direct disruption of mitochondrial metabolism, which includes uncoupling of oxidative phosphorylation and alterations of the components of the electron transport chain which

would lead to changes in mitochondrial membrane potential (MMP)^{60,62}. Changes in MMP results in loss of structural and functional integrity^{60,62}. These changes may occur due to oxidative damage⁷². Damage to mitochondrial DNA also affects mitochondrial function⁶⁰. Mitochondrial dysfunction, in turn, results in the impairment of the cellular energy metabolism which may lead to oxidative stress due to the formation of reactive oxygen species (ROS)^{57,62,71}. Mitochondrial dysfunction can also result in steatosis⁶².

Oxidative stress, the result of a disturbance in the pro-oxidant / anti-oxidant balance of the cell, is another important mechanism of hepatotoxicity⁶⁰. Hepatotoxins capable of generating ROS are usually compounds which are able to undergo repeated cycles of oxidation and reductions such as quinones⁶⁰. Glutathione (GSH), a non-protein thiol, protects the cell from oxidative stress by reducing hydrogen and organic peroxides and also scavenging hydroxyl radicals and singlet oxygens⁷³. Hepatotoxins can deplete the GSH pool by forming GSH-adducts or GSH-conjugates⁷¹. Increased ROS generation and loss of protective thiols, such as GSH, may result in oxidative damage to intracellular biomolecules. In turn, this may alter the structure and function of certain cellular components, such as lipids, resulting in mutation, altered membrane functionality and cellular energetics and ionic homeostasis^{60,71}.

Lipid peroxidation is a common feature of hepatotoxicity, which can be defined as a process mediated by free radicals that result in the oxidative damage of lipids present in cellular membranes⁷⁴. Lipid peroxidation may cause adverse changes to the physicochemical properties of cellular membranes as well as the functionality of enzymes located in these membranes^{74,75}. It may also result in the formation of a large number of degradation products, some of which may be chemically active and result in further cellular dysfunction and possibly death⁶⁰.

Liver injury may also manifest due to steatosis (intracellular accumulation of fat droplets). Steatosis is defined as more than 5% of hepatocytes containing liver fat⁷⁶. Distribution of lipids in hepatocytes can be either microvesicular or macrovesicular⁷⁷. Microvesicular steatosis is an acute impairment of fatty acid β -oxidation resulting from disturbances in the function of mitochondria and ribosomes⁷⁷, Macrovesicular steatosis is more a chronic disturbance of lipid metabolism⁷⁷. Certain xenobiotics can induce

steatosis, which could possibly result in steatohepatitis, and ultimately lead to cirrhosis and liver failure⁷¹.

Structural and/or functional damage to bile canaliculi or bile duct cells, which alter bile flow causes cholestasis and can be a result of accumulation of hepatotoxins^{57,71,78}. Due to accumulation of toxic bile acid and excretory products, cholestasis in turn results in mitochondrial dysfunction and impairment of the anti-oxidant defense system, which again results in further hepatic injury^{57,78}.

1.3 Treatment or reversal of hepatotoxicity

Treatment or reversal of hepatotoxicity is challenging even with all the present day advances in modern medicine. Western medical therapies are often limited in efficacy, produce adverse effects and are expensive⁷⁹. Few compounds exist which provide a hepatoprotective effect or help in the regeneration of hepatic cells⁸⁰. Examples of these include the antioxidant vitamin E⁸¹, sulfhydryl donors such as *N*-acetyl cysteine (NAC)⁸¹⁻⁸³, cysteine, methionine⁸⁴, cysteamine⁸⁴ as well as melatonin⁸¹. Naturally occurring products with hepatoprotective effects include *S*-adenosyl-L-methionine⁸³, polyenylphosphatidylcholine from soyabean extract⁸⁵, and the Shark Bile Salt, 5 β -Scymnol⁸⁶. Corticosteroids and immunosuppressive agents have also been found to bring symptomatic relief⁸⁷. The most commonly used clinical antidote for liver toxicity is NAC, a drug commonly used to treat acetaminophen-induced toxicity. The hepatoprotective effect of NAC can be attributed to a number of mechanisms. One such mechanism is due to its direct binding to the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI)⁸⁶. It also protects against the depletion of essential thiol groups in cellular proteins and enhances GSH synthesis and sulfate conjugation in liver cells⁸⁸. NAC usage is considered reasonably safe, however, it has been classified as a gastrointestinal irritant⁸⁸.

Due to the lack of effective treatment options for hepatotoxicity in modern medicine, alternative therapies are continually being investigated. Medicinal plants and herbal medicine have been used throughout history to treat a vast array of ailments and are

still used in many cultures today^{79,89}. They are being integrated in an ongoing basis with modern medicine as more and more plants are scientifically tested for use in drug therapy. A great number of plants as well as several herbal formulations have been shown to have protective effects against hepatotoxins and it is said that at least a quarter of patients with liver disease use ethnobotanicals⁹⁰. A few examples of plants which have shown to have hepatoprotective effects include *Annona squamosa* (sugar apple), *Chamomile capitula* (chamomile), *Glychirrizia gabra* (liquorice) and *Silybum marianum* (milk thistle)^{79,90}. Of these *Silybum marianum* is probably the best known herbal treatment for hepatotoxicity^{79,91}.

Syzygium cordatum (SC) and *Burkea africana* (BA) are two medicinal plants widely used in Africa with known antioxidant activities and may have possible hepatoprotective effects against the toxicity induced by PCP and its metabolites.

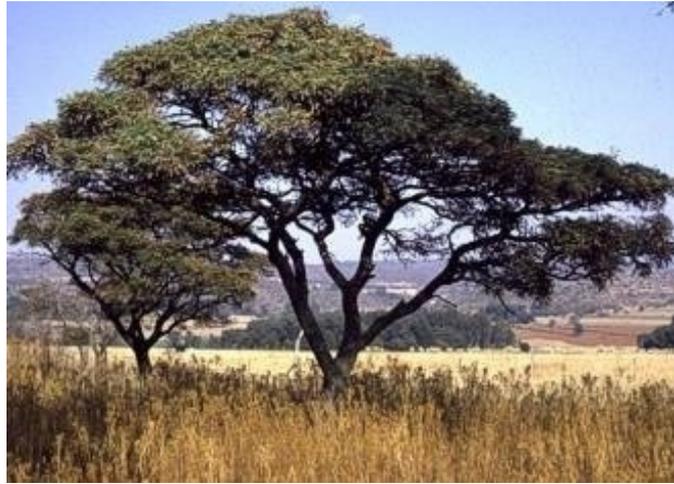
1.3.1 *Burkea africana*

Burkea africana Hook. F (Caesalpinaceae) (Figure 4), commonly known as the Wild Seringa is a deciduous tree found in tropical and sub tropical regions in Africa including Namibia, Botswana and South Africa^{92,93}. It is a medium sized, flat-topped spreading tree that reaches a height of between five and ten meters⁹². Leaves usually have 2-4 pairs of pinnae with each pinnae having 3-18 leaflets per pinnae. Leaflets are oval in shape, grey-green to dark-green and are approximately 30-50 mm in size⁹². Young leaves have a silvery appearance due to oppressed hairs on their surface⁹². Flowers are creamy white, approximately 5 mm in diameter and are produced in long pendulous spikes which are approximately 240 mm in length. The bark is dark grey and has a rough and flaking appearance⁹². Its roots, and more extensively its bark, have found medicinal use in a number of African cultures. The roots are used to treat stomach ache and toothache, whilst the bark is used for treating headache, migraine, dizziness, pain, inflammation and thrush⁹³. It has also found use as an anti-neuralgic as well as a wound-healing and tooth-cleaning agent⁹⁴.

Pharmacologically, BA has not been extensively investigated. It has been reported to possess antioxidant activity^{93,94}. Methanolic as well as dichloromethane extracts of both the bark and fruit have been found to have antifungal and antimolluscicidal activity⁹⁴.

Phytochemical constituents include tannin in the bark and fruit husks as well as flavonoids including proanthocyanidins of which the two major components are said to be fisetinidol-(4 α → 8)-catechin 3-gallate and bisfisetinidol-(4 α →6, 4 α → 8)-catechin 3-gallate⁹³. The bark contains β -sitosterol, Harman-type alkaloids, tryptamine and also monomeric flavan-3-ols(catechin, epicatechin and fisetinidol)⁹³.

A



B



Figure 4. (A) *Burkea africana* Hook. F⁹⁵ (B) Bark of *Burkea africana* Hook. F⁹⁶.

1.3.2 *Syzygium cordatum*

Syzygium cordatum Hochst. ex Krauss (*Myrtaceae*) (Figure 5) commonly known as the Water berry, is an evergreen tree found in moist places or near water from Kwazulu-Natal northwards to Mozambique and also in several other African countries^{97,98}. It is a medium sized, tree that usually reaches a height of between six and fifteen meters⁹⁹. It has a thick, rough, dark brown bark. Many thick, leathery leaves are usually found near the ends of branches where they are arranged in pairs with each pair at almost right angles with the next pair⁹⁹. The leaves can be elliptical, oval or even circular in appearance and are of a bluish green colour on top, being paler green beneath⁹⁹. Creamy white to pink, sweetly scented flowers are found in clusters on the tips of branches and yield abundant nectar^{98,99}. Mainly its bark, but also its leaves and roots have found medicinal use in a number of African cultures¹⁰⁰. It has been used as an emetic and to treat stomach ailments, diarrhea, colds and fever, respiratory ailments and tuberculosis^{98,100}.

Methanol and acetone extracts of the stem-bark and leaves have been found to contain antimicrobial¹⁰¹, antifungal¹⁰⁰ and antioxidant activity¹⁰². Ethyl-acetate leaf extracts have been shown to induce a hypoglycemic effect *in vivo*¹⁰³.

Chemical constituents of SC include friedelin, epi-friedelinol, β -sitosterol, arjunolic acid, gallic acid, ellagic acid, glucose, gallic acid-ellagic acid complex and anthocyanidins such as delphinidin and cyanidin^{100,104}. Tannin has also been found in the sapwood^{100,104}.

A



B



Figure 5. (A) *Syzygium cordatum* Hochst. ex Krauss⁹⁹ (B) Bark of *Syzygium cordatum* Hochst. ex Krauss⁹⁹.

1.4 Detecting hepatotoxicity

Models used to study *in vitro* hepatotoxicity include precision cut tissue slices, isolated perfused organ systems, isolated cells in suspension, established cell lines and primary cell cultures and isolated organelle or enzyme preparations^{61,63}.

Isolated liver cell cultures are probably the most frequently employed hepatic model to investigate hepatotoxicity, of which primary hepatocyte cultures are the most popular^{61,63}. Advantages of isolated liver cultures over perfused organ systems and tissue slices include easy propagation and an extended period of viability¹⁰⁵. They are also more cost effective. They have been used in a large variety of toxicity studies such as cytotoxicity as well as genotoxicity studies, anticancer studies and in metabolic studies^{61,63}. They are also very useful for microscopic investigations of structural changes⁶¹. The HepG2 cell line is one of the most popular immortalized human cell lines employed for hepatotoxicity studies and has been used for studying a large variety of toxins⁶¹.

Cultured hepatocytes have the capacity for biotransformation⁶³. *In vitro* models have been employed to investigate species differences in biotransformation pathways and hepatotoxicity, the mechanism of adverse effects as well as cytotoxic concentrations of test compounds¹⁰⁶⁻¹⁰⁹. *In vitro* liver preparations are increasingly being used as they not only resemble the biotransformation and functional capacity of the liver *in vivo* but also offer varying approaches on all levels of investigational toxicology^{63,106}. The wide range of existing methodologies in which toxicity can be evaluated *in vitro* and the presumption that the toxic effect seen in a whole organism arises fundamentally from cellular dysfunction further supports the use of *in vitro* models¹¹⁰. They have found use in distinguishing whether the parent compound or a metabolite is the hepatotoxic culprit¹⁰⁶. Furthermore, they can be used to test substances for possible hepatoprotective effects¹⁰⁶. *In vitro* models are also used to identify markers for clinical use and for investigating drug interactions, induction and peroxisome proliferation, as well as aiding in the design of further *in vivo* tests¹⁰⁶. Basal cytotoxicity is evaluated by performing proliferation, viability and morphological studies in order to, not only discern whether toxicity is hepatocyte specific, but also to determine the maximal non-toxic

concentration of a test compound^{60,110}. After this, various assays can be employed to detect specific mechanisms of action of the test compound¹¹⁰. Not only are *in vitro* methods useful for detecting various mechanisms of cellular hepatotoxicity, they also to provide information for risk assessment evaluation and to register new chemicals³¹.

The most common of the *in vitro* applications would probably be proliferation, viability and cytotoxicity studies which may include assays such as the 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, Lactate dehydrogenase (LDH) assay and the neutral red uptake (NRU) assay^{111,112}.

Mechanistic hepatotoxic endpoints which can be examined *in vitro* include plasma membrane integrity, cell cycle progression / arrest, mitochondrial physiology (which may include measuring MMP or mitochondrial mass), ROS generation and oxidative stress, cytoskeleton integrity, DNA degradation, apoptosis and necrosis¹¹⁰.

A single assay will never provide a full toxicological profile of a substance⁷¹. For this reason, a number of *in vitro* assays were used to investigate the effects of the test compounds on different aspects of cellular function. This would provide a mechanistic profile of the test compounds, which can be used to hypothesize a mechanism of toxicity. Additionally, it would give some indication of how the medicinal plants and NAC may play a role as hepatoprotective agents, if at all.

1.5 Study Aims

1. Assess organochlorine-induced hepatotoxicity in a mechanistic manner using an in-house developed procedure.
2. Investigate whether methanolic extracts from the bark of BA and SC well as NAC are able to protect against and reverse toxicity induced by PCP and its metabolites.

1.6 Study Objectives

Using the HepG2 cell line:

1. Determine the effect of PCP, TCHQ and TCBQ on :
 - 1.1. Viability using the neutral red uptake assay
 - 1.2. Phase I metabolism by determining CYP1A1 activity using ethoxy-resorufin-*O*-deethylation (EROD) as surrogate
 - 1.3. Oxidative stress by measuring dichlorofluorescein diacetate (DCFDA) cleavage by intracellular ROS
 - 1.4. MMP using JC-1 staining
 - 1.5. Necrosis (as a mode of cell death) by assessing plasma membrane integrity using propidium iodide staining
 - 1.6. Apoptosis (as a mode of cell death) by assessing caspase-3 activity
2. Treat cells with plant extracts or a known hepatoprotective agent prior to organochlorine exposure to assess possible protection against the induced toxicity.

Chapter 2- Materials and Methods

2.1 Chemicals and Equipment

A list of chemicals used and their suppliers are found in Annexure B. A list of equipment used and its manufacturers are found in Annexure C.

2.2 Cells and cell maintenance

HepG2 (ATCC catalogue number HB-8065), a human hepatocarcinoma cell line, was purchased from the American Tissue Culture Collection. Ethical approval for the use of this commercially available cell line was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (Annexure A). The HepG2 cell line has a variety of liver specific metabolic functions and was chosen especially for its CYP expressing capability¹¹³.

HepG2 cells were maintained in EMEM, supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% heat-inactivated fetal calf serum at 37 °C in a 5% carbon dioxide atmosphere.

Cells were seeded into 96-well microtiter culture plates at a density of 2×10^4 cells/well and incubated for 48h at 37°C with 95% humidity and 5% CO₂ to acclimatise. The density of viable cells was determined microscopically using the trypan blue exclusion method¹¹⁴.

2.3 Mechanistic assays and plate set up

In the present study viability (columns 1 + 2), CYP1A1 activity (columns 3 + 4), necrosis (columns 5 +6), ROS generation (columns 7 + 8), MMP (columns 9 +10) and apoptosis (columns 11 + 12) were determined as endpoints using a single plate method developed in-house¹¹⁵ (Figure 3).

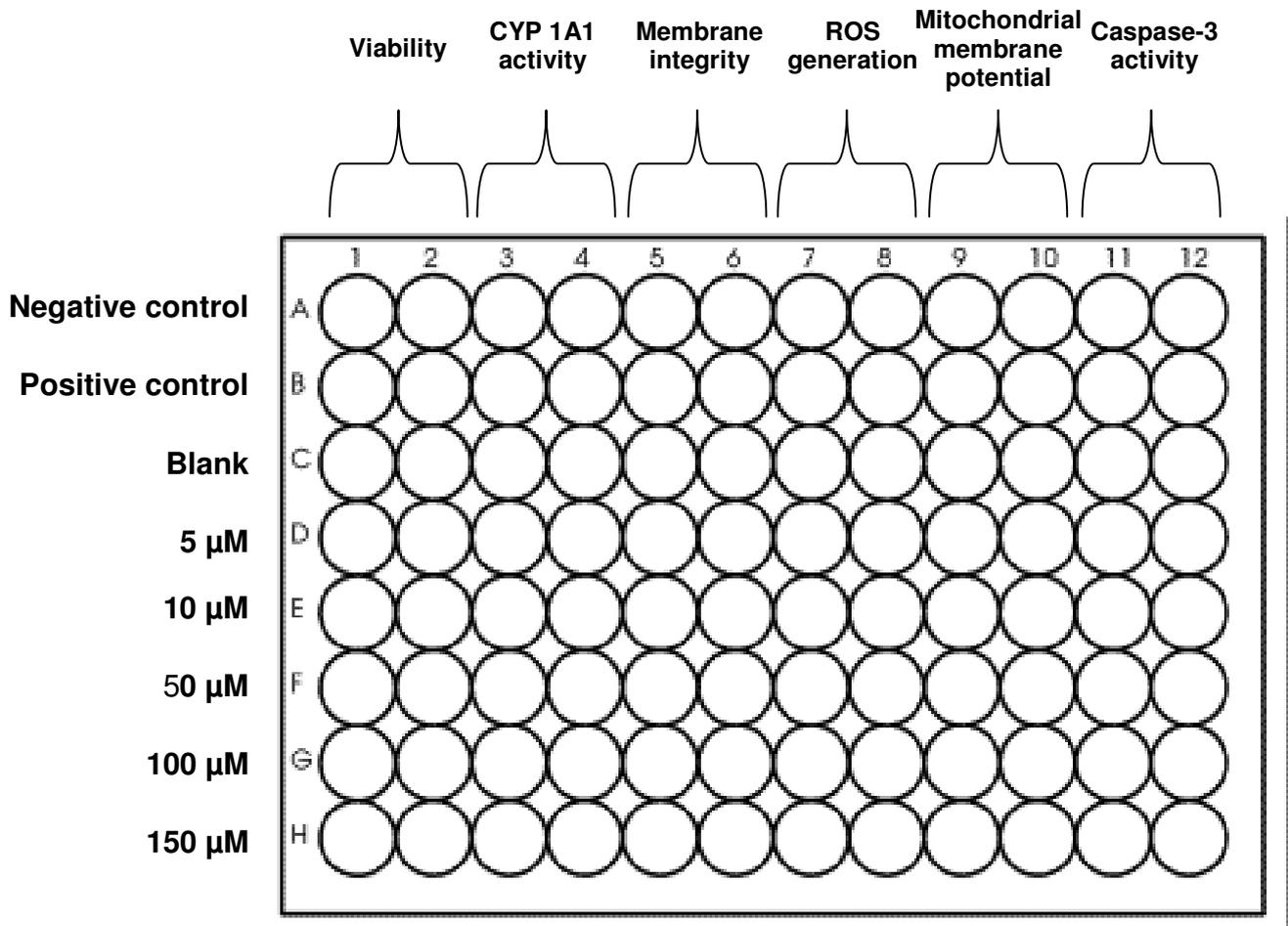


Figure 6. Diagram of the plate setup for a 96-well microtitre plate.

Viability was measured to determine at which concentrations PCP and its metabolites adversely affect cell survival. CYP1A1 activity was determined as it is involved in the metabolic activation and inactivation of many environmental contaminants and chemical carcinogens¹¹⁶.

ROS generation was determined because oxidative stress is an important mechanism of hepatotoxicity and is said to be a frequent mediator of cell death¹¹⁷. The 2',7'-dichlorofluorescein diacetate (DCFDA) assay is a common fluorometric assay used for quantitation of ROS in cells. Non-ionic, non-polar DCFH-DA is able to penetrate the cell membrane of intact cells, and is hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescein DCFH^{118,119}. DCFH is further oxidized to highly fluorescent DCF^{118,119}. This fluorescence can then be quantified.

MMP was determined since it is an important indicator of mitochondrial homeostasis. The latter has an immediate effect on energy homeostasis and, therefore, also cell viability^{71,120}. Positively charged JC-1 enters the negatively charged mitochondria of healthy cells where it forms aggregates which emit red fluorescence at excitation 544 nm and emission 590 nm¹²¹. A high MMP is detected by a high concentration of JC-1 in the mitochondria which results in aggregate formation. Some JC-1 is also found in the cytosol of healthy cell in monomeric form which emits green fluorescence at 485 nm and emission 520 nm. If the MMP collapses, the positively charged dye is then distributed throughout the cell in a monomeric form which emits green fluorescence at 485 nm and emission 520 nm¹²¹. Therefore the ratio of red fluorescence/green fluorescence can be used as an indication of the state of the MMP.

Necrosis and apoptosis were investigated to determine the mode of cell death. Membrane integrity was assessed as it is a good indicator of necrosis. Propidium iodide is an impermeable fluorescent molecule and can thus only enter dead or dying cells with compromised cellular membranes¹²². It intercalates into DNA where its fluorescence is enhanced. Increased fluorescence therefore indicates compromised cellular membrane integrity, which suggests necrotic cell death. Caspase-3 activity was determined as it indirectly measures the number of cells committed to apoptosis¹²³. The assay is based on the hydrolysis of a peptide substrate acetyl-asp-7-amino-4-methylcoumarin by

caspase-3, which releases fluorescent 7-amino-4-methylcoumarin (AMC) after cleavage. Greater fluorescence indicates higher caspase-3 activity indicative of apoptotic cell death.

These end points and their effect on hepatotoxicity are presented in Figure 7.

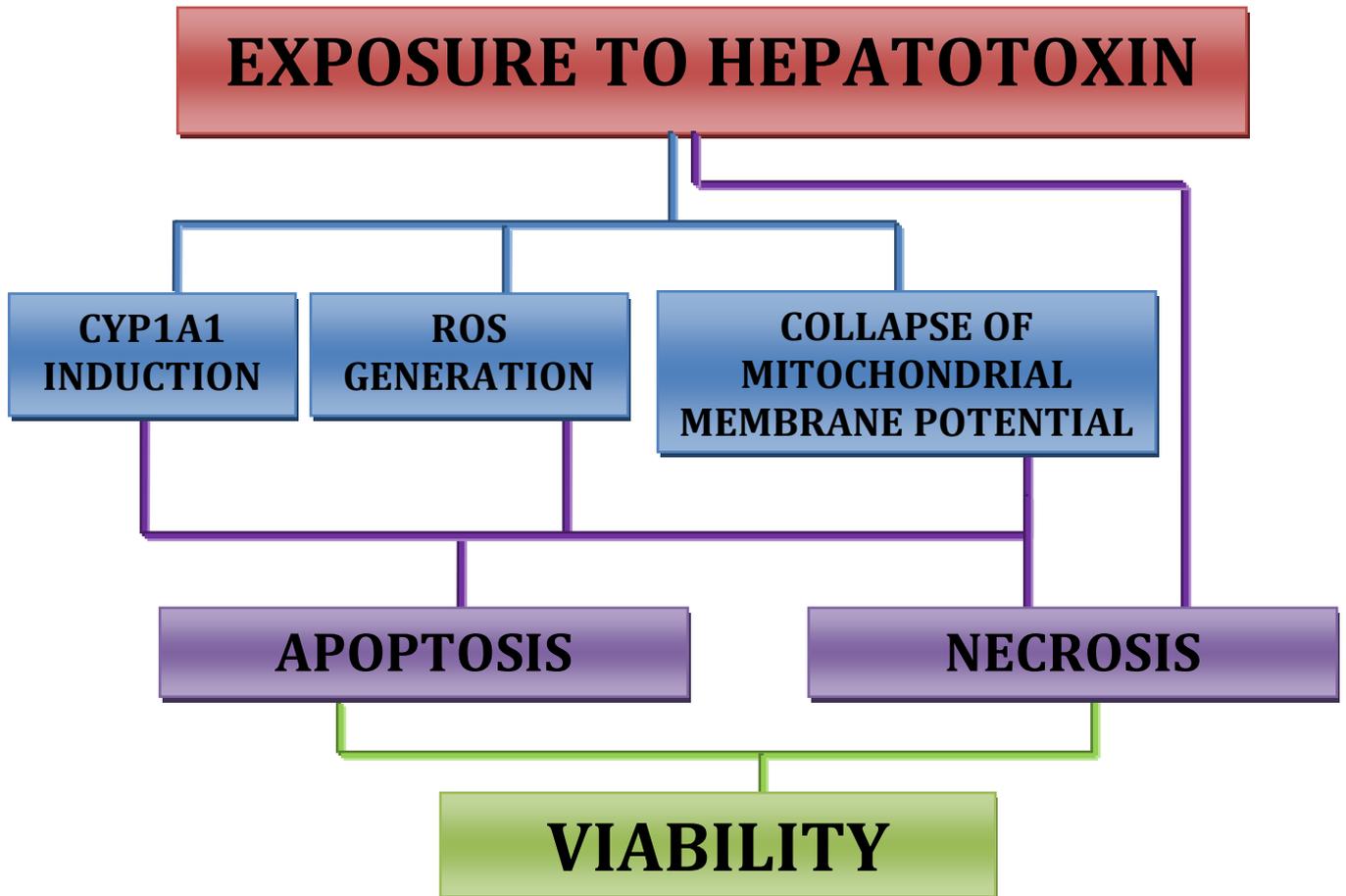


Figure 7. Diagram depicting the various hepatotoxic endpoints which were evaluated.

2.4 Mechanistic studies

In all multiplex experiments cells were plated (100 μ l) at a density of 2×10^4 cells/well. Cells were treated with 100 μ l of 10, 20, 50, 200 and 300 μ M of PCP or its metabolites. Final concentrations of compounds in the wells for all assays amounted to 5, 10, 50, 100 and 150 μ M. Incubation / exposure took place at 37°C in 95% humidity and 5% CO₂, unless otherwise stated.

2.4.1 Determination of cell viability

Cell viability was assessed using the NRU assay adapted from Fotakis and Timbrell¹¹². Cells were exposed to PCP or one of its two metabolites for 24 h. After exposure, medium was removed and cells incubated with 100 μ g/ml neutral red dye dissolved in EMEM (pH 6.4) for 2 h. Cells were then washed with PBS and dried over night, after which 100 μ l of elution medium (EtOH/AcCOOH/H₂O, 49:1:50 (v/v/v)) was added. The plates were put on an orbital shaker for 30 min to aid dye dissolution and spectrophotometrically read at 540 nm using a ECX 800 universal plate reader. Tamoxifen (150 μ M) was used as positive control.

2.4.2 Determination of CYP 1A1 activity

CYP1A1 activity was assessed using the ethoxy-resorufin-*O*-deethylation method. Cells were exposed to PCP or one of its two metabolites for 24 h. Following exposure, medium was removed and cells incubated with 100 μ l of serum-free medium containing 150 nM 7-ethoxyresorufin for 2 h after which fluorescence was measured at 544 nm excitation and 590 nm emission using a FluoStar Optima Fluorescent plate reader. Omeprazole (150 μ M) was used as positive control.

2.4.3 Determination of reactive oxygen species generation

This assay was performed using the method of Zhang *et al.*¹²⁴. Cells were preloaded with 5 μ M DCFDA for 1 h, after which the cells were washed with PBS and exposed to PCP or one of its two metabolites for 3 h. Fluorescence was monitored at excitation 485 nm and emission 520 nm using a FluoStar Optima Fluorescent plate reader. AAPH (150 μ M) was used as positive control.

2.4.4 Determination of mitochondrial membrane potential

Cells were loaded with 20 μM JC-1 for 1 h. Thereafter the cells were washed in PBS and exposed to PCP or one of its two metabolites for 2 h. Fluorescence was monitored at excitation 485 nm and emission 520 nm for the monomeric form and excitation 544 nm and emission 590 nm for the aggregate form using a FluoStar Optima Fluorescent plate reader. Tamoxifen (150 μM) was used as positive control.

2.4.5 Determination of necrosis by assessing cell membrane integrity

This assay was performed using the method adapted from Nieminen *et al*¹²⁵. Cells were exposed to PCP or one of its two metabolites for 24 h. After exposure, medium was removed and the cells incubated with 15 μM PI for 5 min, after which cells were washed in PBS. Fluorescence was monitored at excitation 544 nm and emission 620 nm using a FluoStar Optima Fluorescent plate reader. 0.5% Triton X-100 was used as positive control and was added 30 min prior to staining with PI.

2.4.6 Determination of apoptosis via Caspase-3 activity

The caspase-3 assays were performed in order to investigate apoptosis.

The incomplete lysis buffer contained 10 mM HEPES (pH 7.5), 2 mM EDTA and 5 mM CHAPS. The complete lysis buffer was made up by addition of 4 μl 2-mercaptoethanol, 100 μl 50 mM PMSF to every 10 ml of incomplete lysis buffer. The incomplete assay buffer contained 20 μM HEPES (pH 7.5) and 2 mM EDTA. The complete assay buffer was made up by addition of 4 μl 2-mercaptoethanol, 100 μl 50 μM PMSF and 5 μl 10mM caspase-3 substrate to every 10 ml incomplete assay buffer.

Cells were exposed to PCP or one of its two metabolites for 6h. After exposure plates were put on ice for at least 15 min. Thereafter 25 μl ice-cold complete lysis buffer was added and incubated for a further 15 min on ice. Complete assay buffer (100 μl) was then added and plates incubated overnight. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a FluoStar Optima Fluorescent plate reader. Staurosporine (11 μM) was used as positive control.

2.5 Determination of effect of plant extracts on mechanistic parameters

2.5.1 Plant material

BA (voucher no. LT 15) is lodged at the herbarium in the Department of Toxicology, Onderstepoort Veterinary Institute (Pretoria). Its identity was confirmed by the South African National Biodiversity Institute (Pretoria). SC (voucher no. NH 1880) was collected and identified by DR. N Hahn and is lodged at the Soutpansbergensis herbarium.

2.5.2 Preparation of methanolic plant extracts

The dried bark of BA and SC were ground to a powder. Of this powder, 1.0 g was added to 10 ml of methanol. The mixture was sonicated for 30 min and incubated overnight at 4°C. The extract was then centrifuged at 1000 *g* for 10 min, after which the supernatant was removed, filtered through a 0.45 µm filter and dried using a rotavapor before being resuspended in distilled water and lyophilized. Yields were determined gravimetrically. Prior to *in vitro* use, the extract was dissolved in culture medium using 0.5% dimethyl sulfoxide (DMSO) and then filter sterilized (0.22 µM).

2.5.3 Mechanistic parameters

In order to investigate whether BA, SC and NAC have possible protective effects against PCP and its metabolites, the mechanistic assays described in 2.4.1 - 2.4.6 were repeated after pre-treating the cells with either 100 µg/ml methanolic extracts from the bark of BA or SC, or 100 µg/ml NAC for 1 h at 37°C in 95% humidity and 5% CO₂.

2.6 Statistical analyses

For viability, IC_{50} values were determined from dose-response curves produced by fitting observed values to a Hill equation with variable slope using Graphpad Prism 4.0.

Grubb's test for outliers was performed in order to exclude outliers.

Significant differences between various groups were detected by performing either unpaired T-test or Mann Whitney tests depending on the normality of the data using Graphpad Prism 5.0. The Kolgomorv Smirnov test was performed to test the normality of the data (Graphpad Prism 5.0).

Three repeats of three plates each were run on at least three different occasions for PCP, TCHQ and TCBQ in the mechanistic studies. For screening of plant extracts, tests were performed in triplicate.

All results, except IC_{50} values are expressed as a % mean \pm SEM of the respective vehicle control, unless stated otherwise.

Significant difference from the vehicle control are indicated by *, ** and *** for p values < 0.05, 0.01 and 0.001, respectively. Significant differences between groups exposed to pesticides alone and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• for p values < 0.05, 0.01, 0.001, respectively

Chapter 3 - Results

3.1 Mechanistic studies

3.1.1 Cell viability

Neutral red is a supravital dye that accumulates in the lysosomes of viable cells^{112,126}. A decrease in NRU is a direct indication of the cytotoxicity of a substance.

IC₅₀ values for PCP, TCHQ and TCBQ are presented in Figure 8. PCP was most toxic followed by TCBQ and lastly TCHQ.

PCP showed a dose-dependent increase in cytotoxicity (Figure 9A). Significant decreases ($p < 0.01$) in viability compared to the vehicle control were observed in cells exposed to 50, 100 and 150 μM PCP. Both TCHQ and TCBQ showed negligible cytotoxic effect at concentrations between 5 and 100 μM . A dose-dependent, significant ($p < 0.01$) increase in cytotoxicity, compared to the vehicle control, at a concentration of 150 μM was detected for both metabolites. (Figure 9 B and C).

Tamoxifen was used as a positive control as it has been found to cause cell death in HepG2 cells¹²⁷. A bar graph of the pooled results for viability of cells exposed to Tamoxifen compared to the pooled results for viability of vehicle control cells is provided in Figure 10. Cells exposed to Tamoxifen showed a significant ($p < 0.001$) decrease in cell viability compared to vehicle control cells showing that the assay performed as expected. Cells treated with Tamoxifen had a cell viability of $14.76 \pm 1.22\%$ that of the cell viability in the vehicle control.

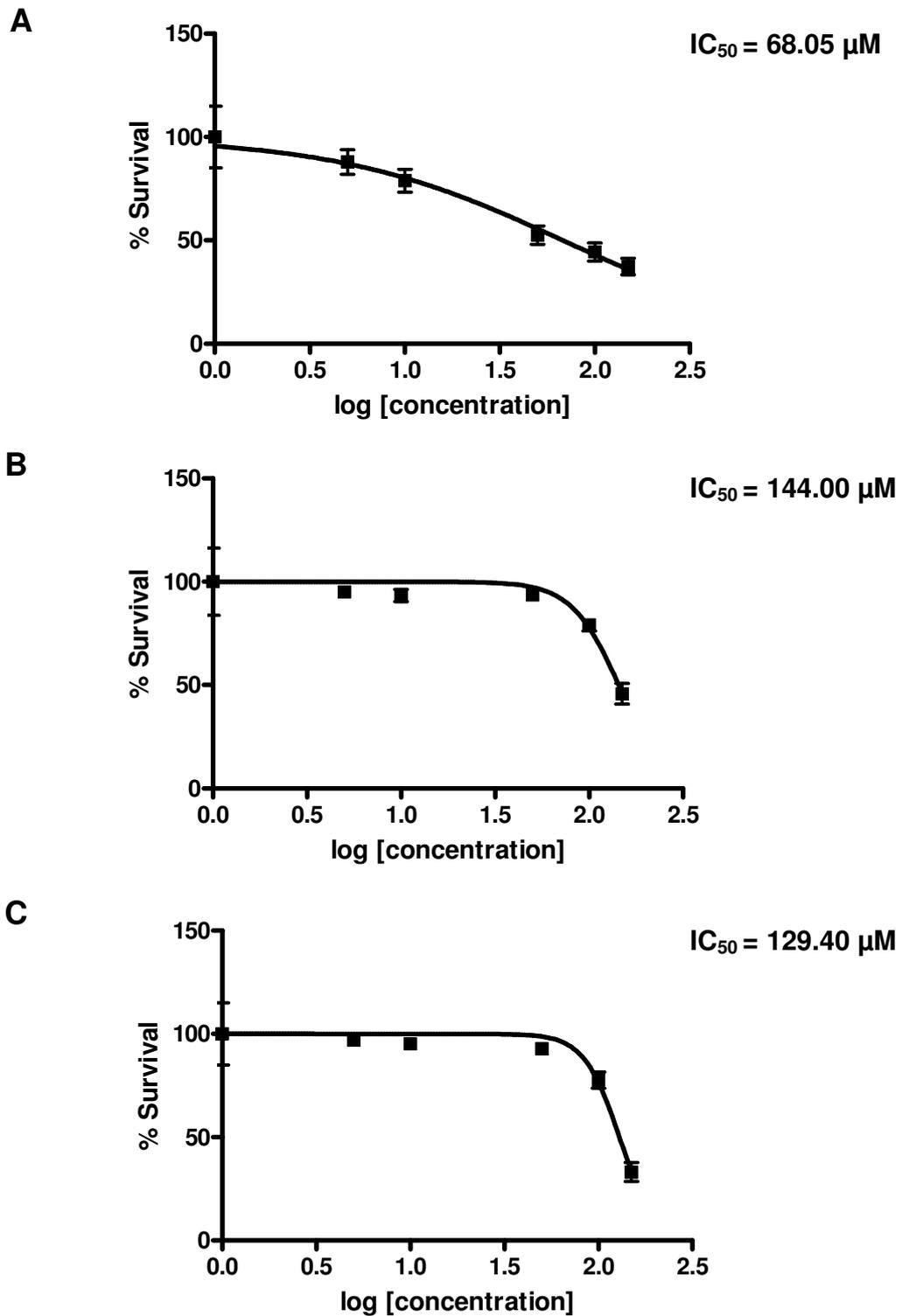


Figure 8. Survival curves of HepG2 cells after 24 h exposure to (A) PCP, (B) TCHQ and (C) TCBQ.

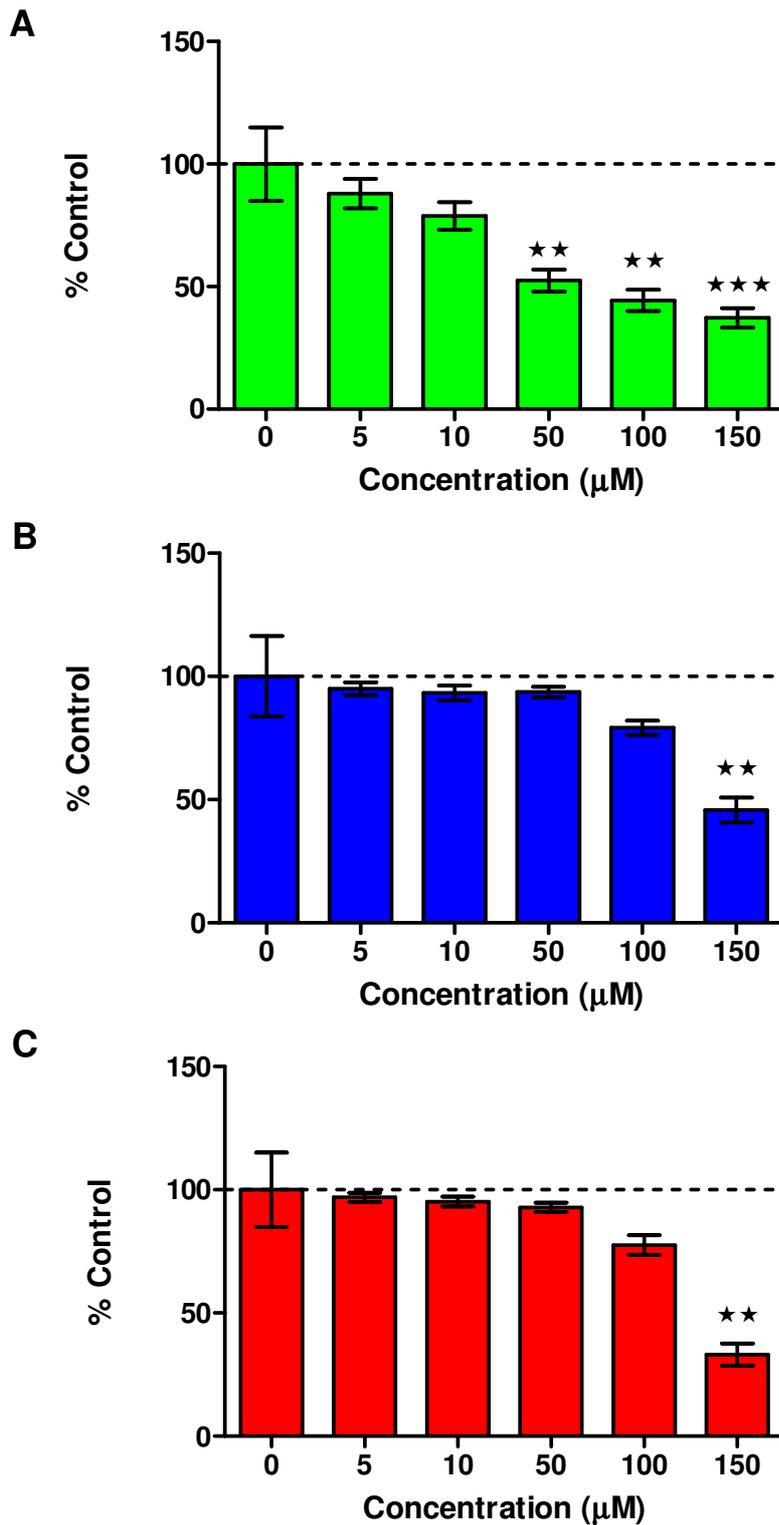


Figure 9. Cell viability in HepG2 cells exposed for 24 h to **(A)** PCP, **(B)** TCHQ or **(C)** TCBQ. Significant difference from the vehicle control is indicated by ** and *** representing p values < 0.01 and 0.001, respectively.

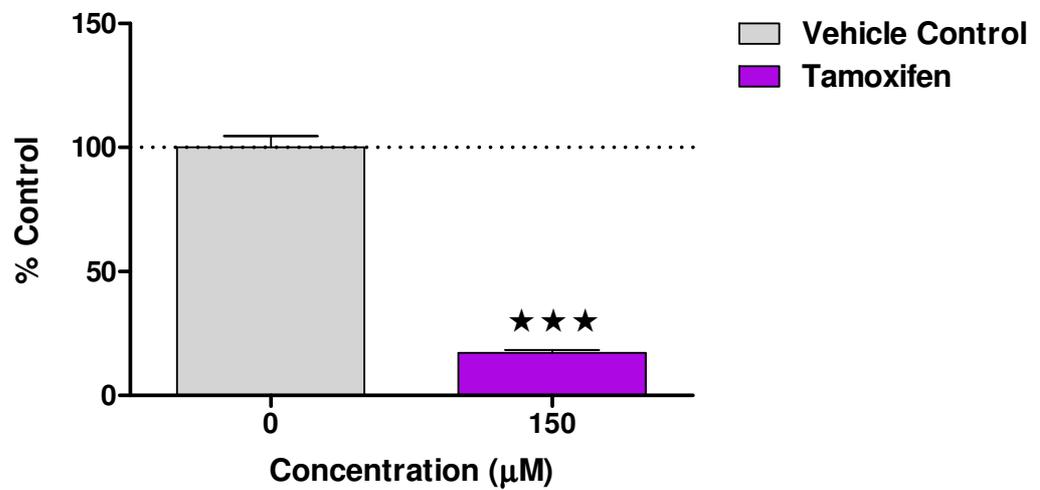


Figure 10. Cell viability in HepG2 cells exposed for 24 h to the positive control, Tamoxifen, compared to viability in vehicle control cells. Significant difference from the vehicle control is indicated by ***, p value < 0.001.

3.1.2 CYP1A1 activity

Activities of CYP1A1 were measured as a rate of the *O*-dealkylation of 7-ethoxyresorufin to yield highly fluorescent resorufin. Greater fluorescence indicates greater CYP1A1 activity.

Of the three compounds tested, PCP caused the greatest induction of CYP1A1 in HepG2 cells. Increases were however not dose-dependent. Cells treated with concentrations of 5 to 100 μ M PCP showed significantly ($p < 0.05$) higher CYP 1A1 activity than that observed in the vehicle control (Figure 11A). Although not significant, a decrease in CYP1A1 activity was observed in cells exposed to 150 μ M PCP where CYP1A1 activity was only 64.69 ± 14.85 % of the CYP1A1 activity observed in the vehicle control.

Dose-dependent changes in CYP1A1 activity occurred in cells exposed to TCHQ and TCBQ. TCHQ at a concentration of 50 μ M increased CYP 1A1 significantly ($p < 0.001$) where CYP1A1 activity was 192.10 ± 17.12 % of the control. No significant increase in CYP1A1 activity was observed in cells exposed to 5, 10 and 100 μ M TCHQ whilst a significant decrease in CYP1A1 activity was seen in cells exposed to 150 μ M TCHQ ($p < 0.01$) (Figure 11B). No significant change in CYP1A1 activity was observed in cells exposed to 5 and 10 μ M TCBQ. Significant ($p < 0.001$) CYP1A1 induction was seen in cells exposed to 50 and 100 μ M TCBQ whilst a significant ($p < 0.001$) decrease in CYP1A1 was observed in cells exposed to 150 μ M TCBQ (Figure 11C).

Omeprazole was used as a positive control as it has been shown to induce CYP1A1 activity in HepG2 cells¹²⁸. Figure 12 depicts the pooled results for CYP1A1 activity in cells exposed to 150 μ M omeprazole compared to the pooled results of CYP1A1 for vehicle control cells. Omeprazole produced a significant ($p < 0.001$) increase in CYP1A1 activity compared to that observed in the vehicle control indicating that the assay performed as expected. CYP1A1 activity in cells exposed to omeprazole was 141.70 ± 28.64 % of the CYP 1A1 activity observed in the vehicle control.

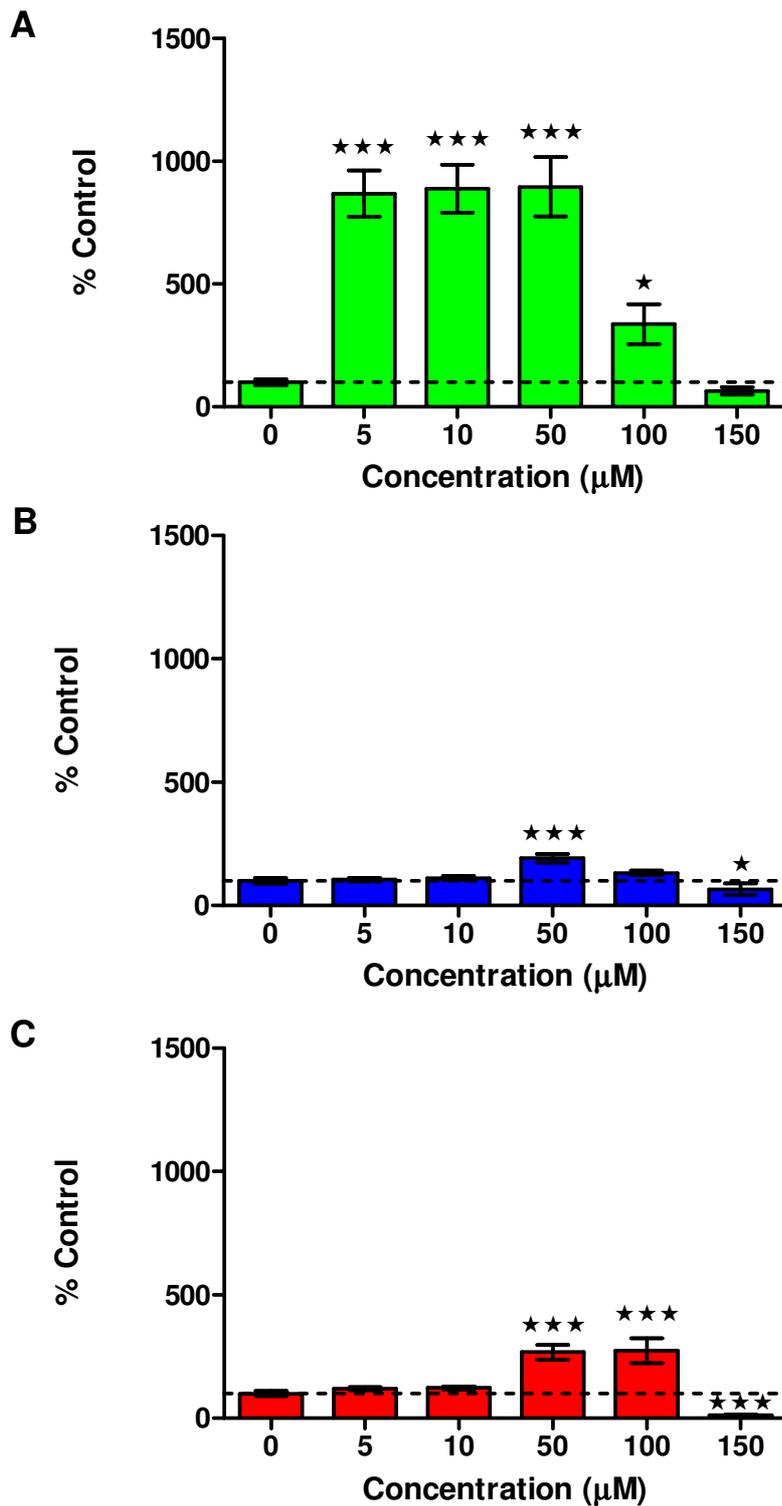


Figure 11. CYP1A1 activity observed in HepG2 cells after 24 h exposure to **(A)** PCP, **(B)** TCHQ or **(C)** TCBQ. Significant difference from the vehicle control is indicated by * and *** representing p values < 0.05 and 0.001, respectively.

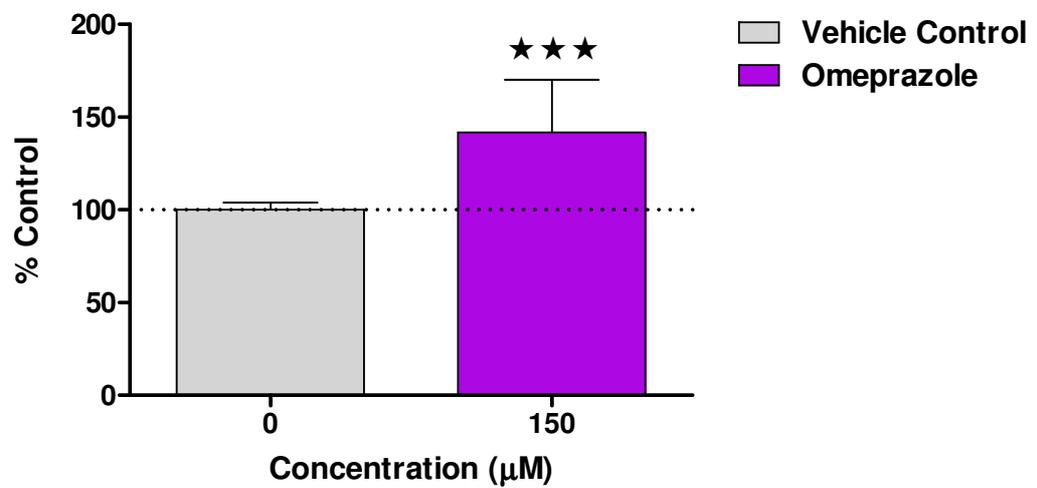


Figure 12. CYP1A1 activity observed in HepG2 cells exposed for 24 h to the positive control, Omeprazole compared to CYP1A1 activity in vehicle control cells. Significant difference from the vehicle control is indicated by *******, p values < 0.001.

3.1.3 Reactive oxygen species generation

ROS generation was determined by measuring DCFDA cleavage by H_2O_2 to highly fluorescent DCFH¹²⁹. Fluorescence is therefore directly proportional to the concentration of hydrogen peroxide in the cells and gives an indication of ROS generation in the cell.

In HepG2 cells exposed to PCP, no significant ROS generation was observed (Figure 13A). When HepG2 cells were exposed to TCHQ, significant ($p < 0.001$) ROS generation was noted at most test concentrations except at 5 μM where the increase in ROS was not significant (Figure 13B). ROS generation in cells exposed to TCHQ followed a dose response. Cells exposed to TCBQ showed significant ($p < 0.001$) ROS generation for all test concentrations (Figure 13C). These increases, however, did not follow a dose-response pattern.

AAPH was used as a positive control. It is known to elicit ROS generation in HepG2 cells¹³⁰. The positive control, AAPH showed significant ($p < 0.001$) ROS generation compared to vehicle controls (Figure 14). The latter confirmed that the assay performed as expected.

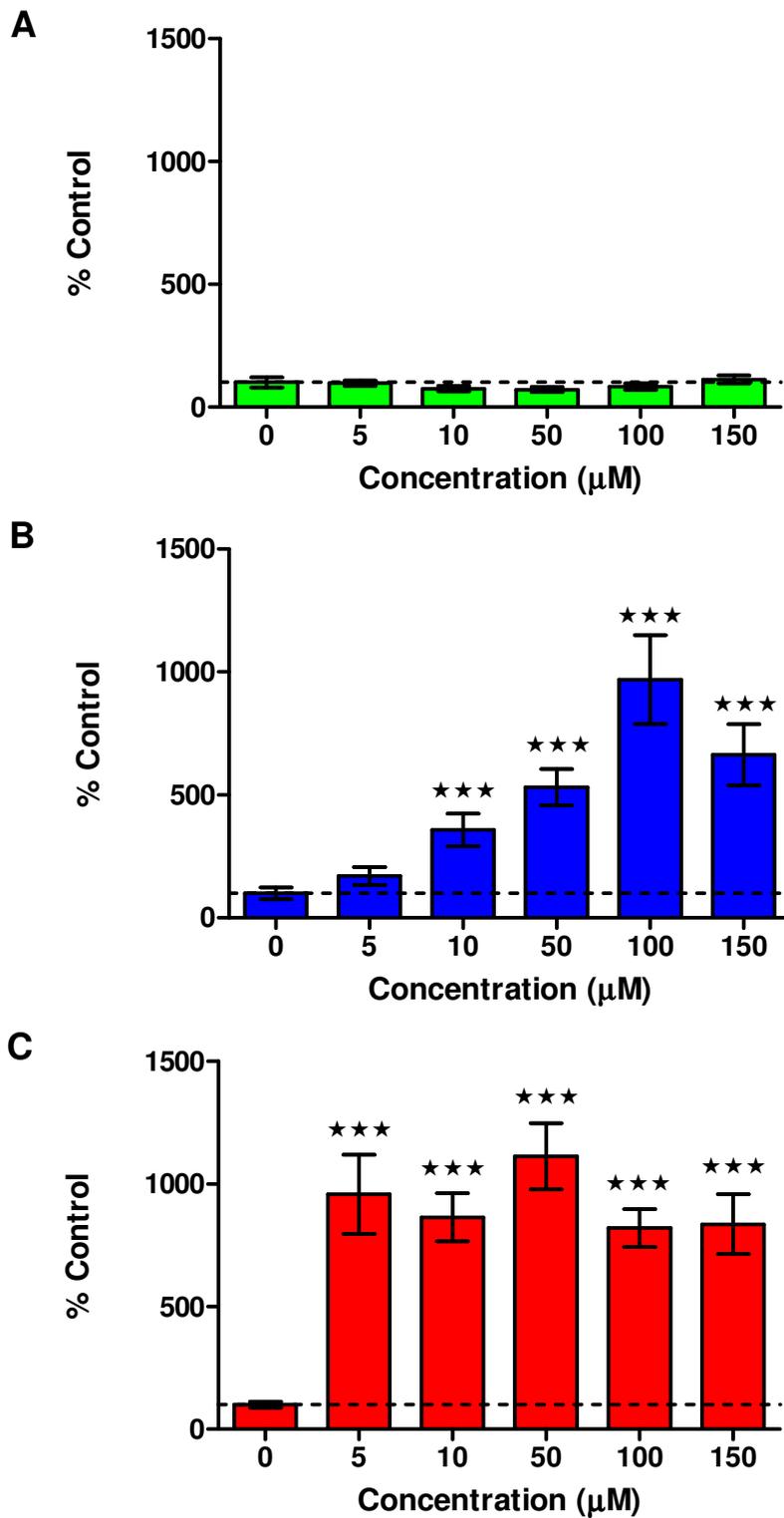


Figure 13. ROS generation observed in HepG2 cells following 3 h exposure to (A) PCP, (B) TCHQ or (C) TCBQ. Significant difference from the vehicle control is indicated by ***, p values < 0.001.

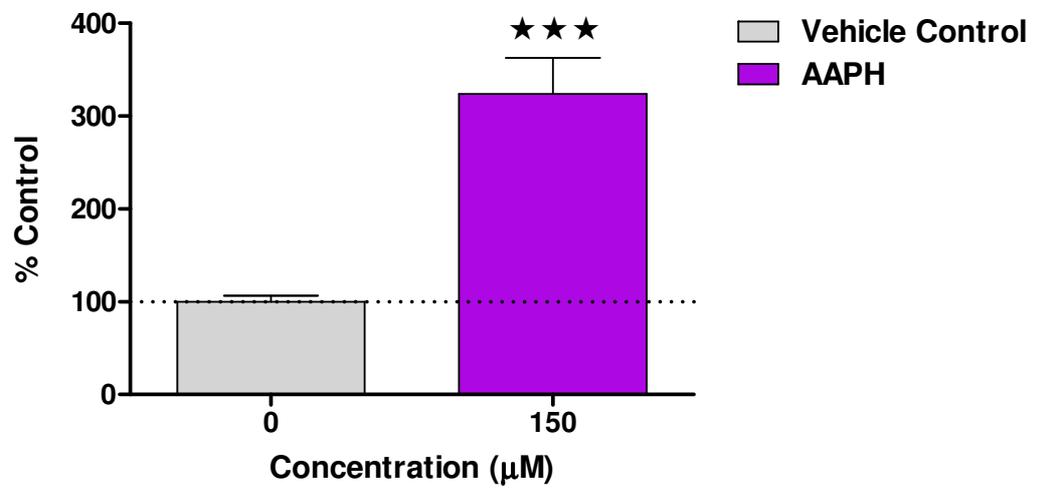


Figure 14. ROS levels observed in HepG2 cells exposed for 3 h to the positive control, AAPH, compared to ROS levels in vehicle control cells. Significant difference from the vehicle control is indicated by ***, p values < 0.001.

3.1.4 Mitochondrial membrane potential

The ratio of red fluorescence to green fluorescence (indicative of MMP) for cells exposed to PCP, TCHQ and TCBQ are presented in Figure 15.

PCP caused significant ($p < 0.05$) mitochondrial depolarization (decreased red/green ratio) in HepG2 cells at 5 and 10 μM concentrations (Figure 15A). Both TCHQ and TCBQ caused significant ($p < 0.05$) mitochondrial depolarization at concentrations $\geq 10\mu\text{M}$ (Figure 15B and C). A maximum response in MMP occurred for both TCHQ and TCBQ between 50 and 150 μM .

A bar graph of the pooled results for MMP in cells exposed to Tamoxifen, the positive control, compared to the pooled results of MMP for vehicle control cells is depicted in Figure 16. Tamoxifen has been shown to decrease MMP in HepG2 cells¹³¹. A significant ($p < 0.001$) decrease in MMP was seen in cells treated with Tamoxifen compared to vehicle treated cells, indicating that the assay performed satisfactory.

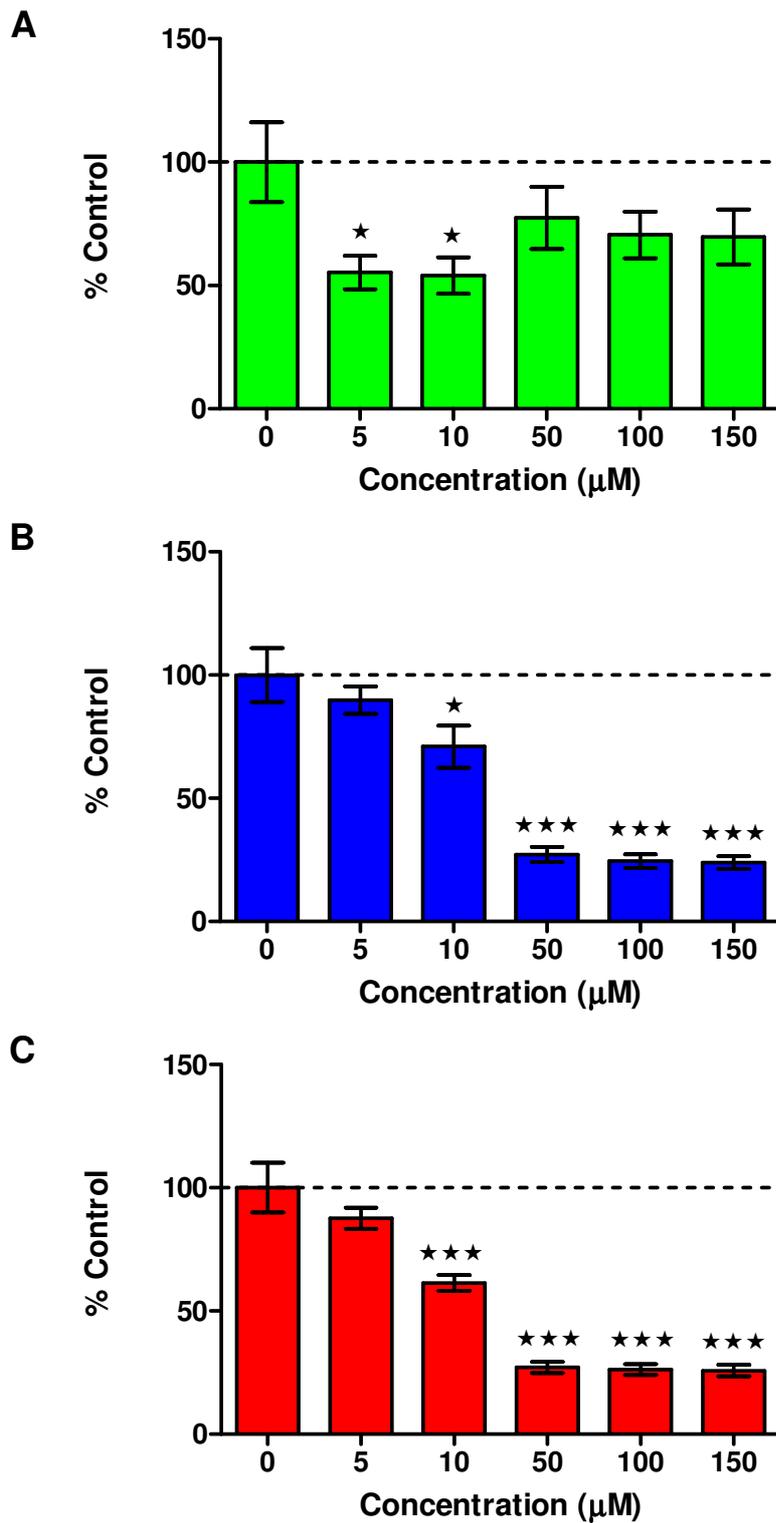


Figure 15. Mitochondrial membrane potential observed in HepG2 cells after 1 h exposure to **(A)** PCP, **(B)** TCHQ or **(C)** TCBQ. Significant differences from the vehicle control is indicated by * and *** representing p values < 0.05 and 0.001, respectively.

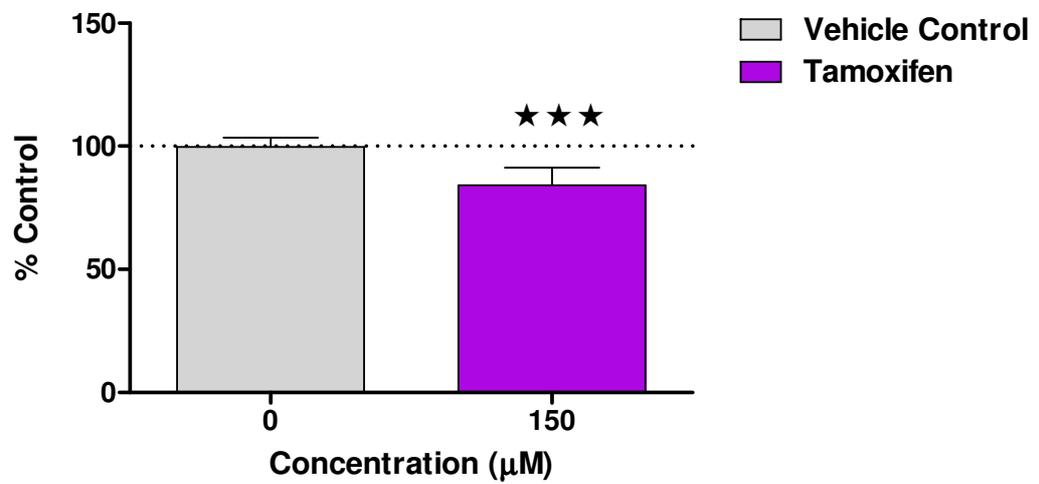


Figure 16. MMP observed in HepG2 cells exposed for 1 h to the positive control, Tamoxifen, compared to the MMP in vehicle control cells. A significant difference from the vehicle control is indicated by *******, p values < 0.001.

3.1.5 Necrosis

Although small increases were present in PI fluorescence, the majority were not significant whether cells were exposed to either PCP or its metabolites (Figure 17). The only significant ($p < 0.05$) decrease in PI fluorescence was in cells treated with 150 μM of either TCHQ and TCBQ

Triton X-100 was used as a positive control. Triton X100 is a known non-ionic surfactant which permeabilizes cell membranes thus leading to necrosis¹²⁶. A significant ($p < 0.001$) increase in PI fluorescence was seen in cells exposed to a 0.5% (v/v) Triton X-100, compared to vehicle control treated cells showing that the assay performed as expected (Figure 18).

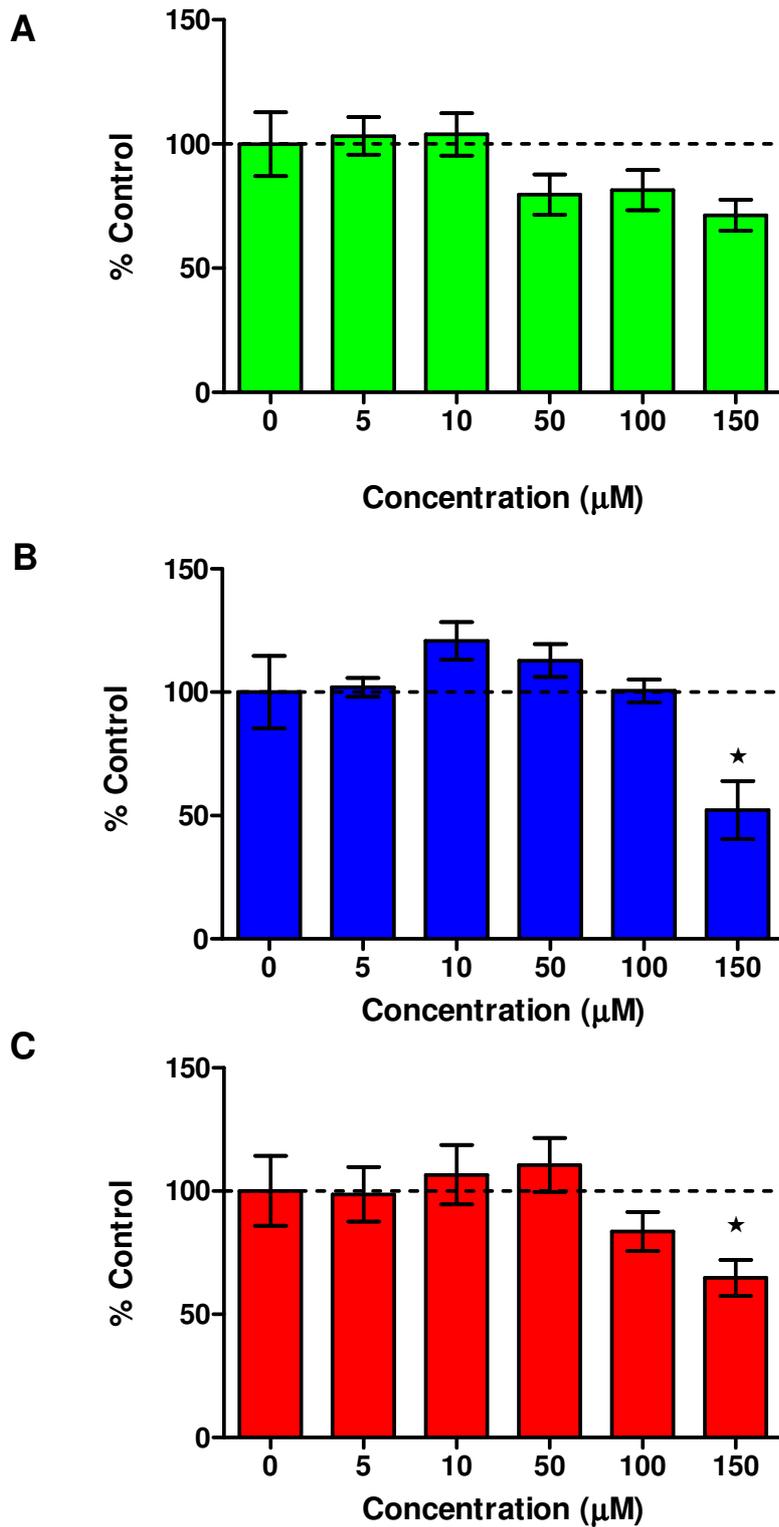


Figure 17. Membrane integrity as a measure of necrosis determined by PI staining in cells after 24 h exposure to **(A)** PCP, **(B)** TCHQ or **(C)** TCBQ. Significant difference from the vehicle control is indicated by *, p values < 0.05.

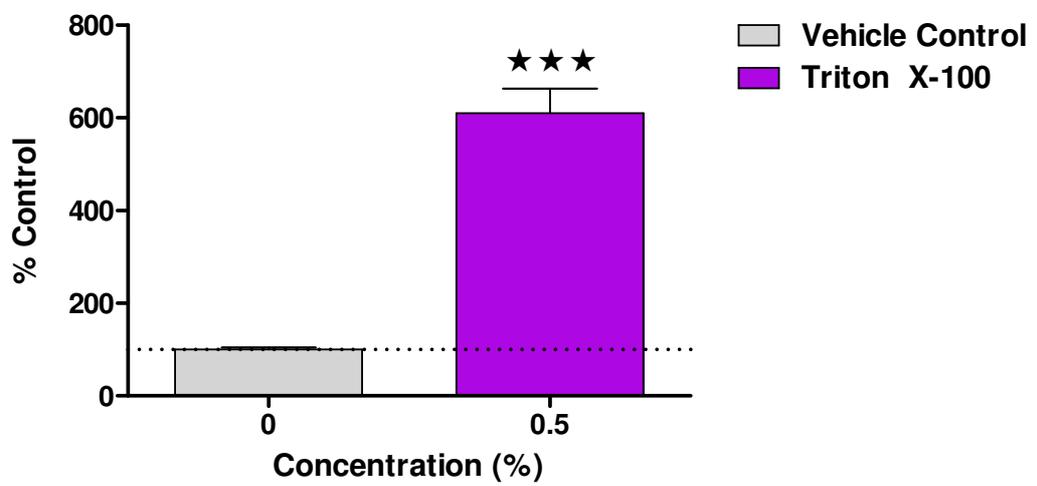


Figure 18. Necrosis as determined by PI staining in HepG2 cells exposed to the positive control, Triton X-100, compared to that of the vehicle control. A significant difference from the vehicle control is indicated by $***$, p values < 0.001 .

3.1.6 Apoptosis

No significant increase in caspase-3 activity was observed in cells exposed to either PCP or its metabolites (Figure 19). A significant decrease, $p < 0.05$ and $p < 0.001$, in caspase-3 activity was observed in cells exposed to 100 and 150 μM TCHQ, respectively (Figure 19B). TCBQ had no significant effect on caspase-3 activity (Figure 19C).

A significant increase in caspase-3 activity was seen in cells treated with 11 μM of the positive control, staurosporine, compared to vehicle controls (Figure 20). Staurosporine is known to cause apoptosis¹³². The results therefore indicate that the assay performed as expected.

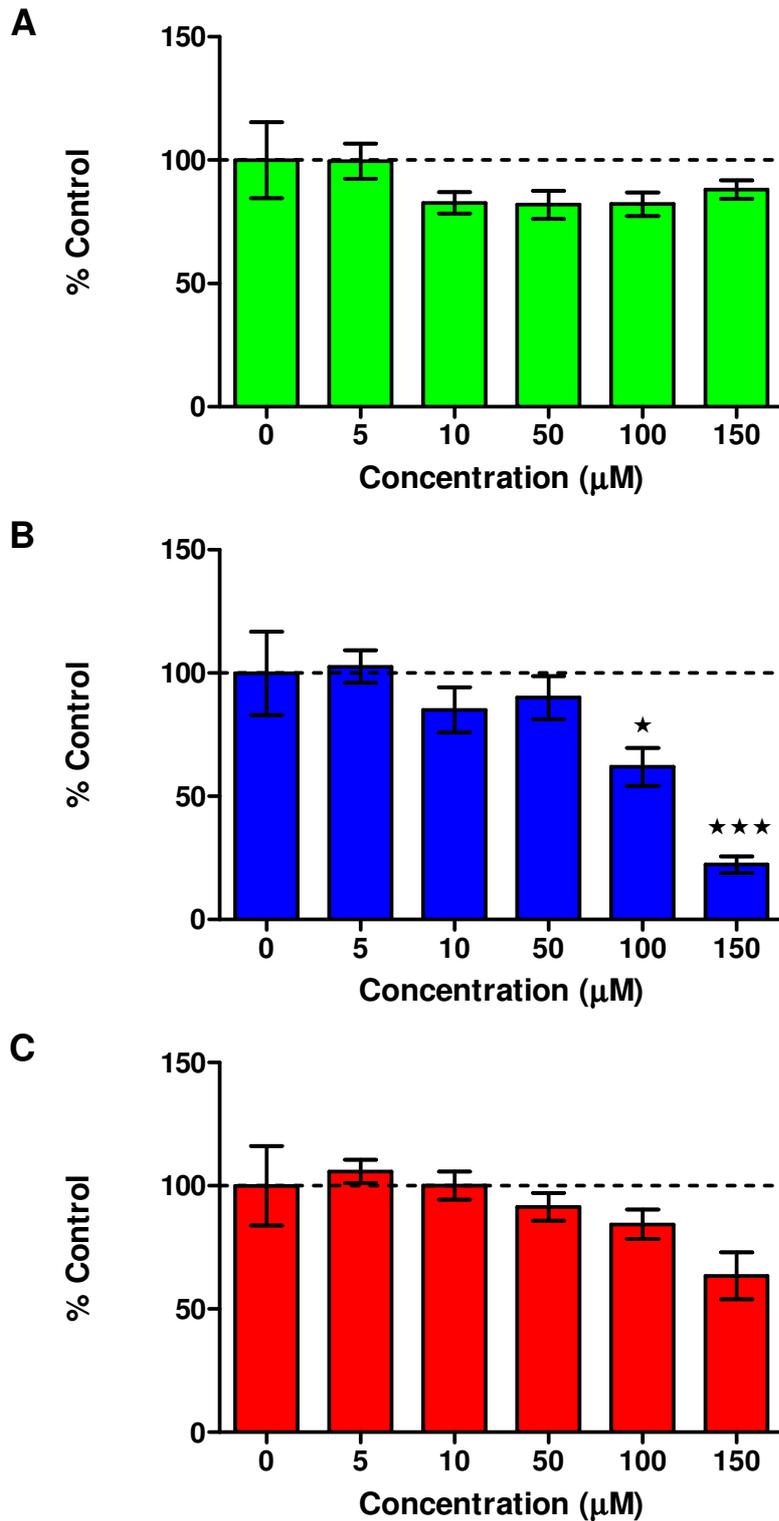


Figure 19. Caspase-3 activity as an indication of apoptosis observed in HepG2 cells after 6 h exposure to **(A)** PCP, **(B)** TCHQ or **(C)** TCBQ. Significant difference from the vehicle control is indicated by * and *** representing p values < 0.05 and 0.001, respectively.

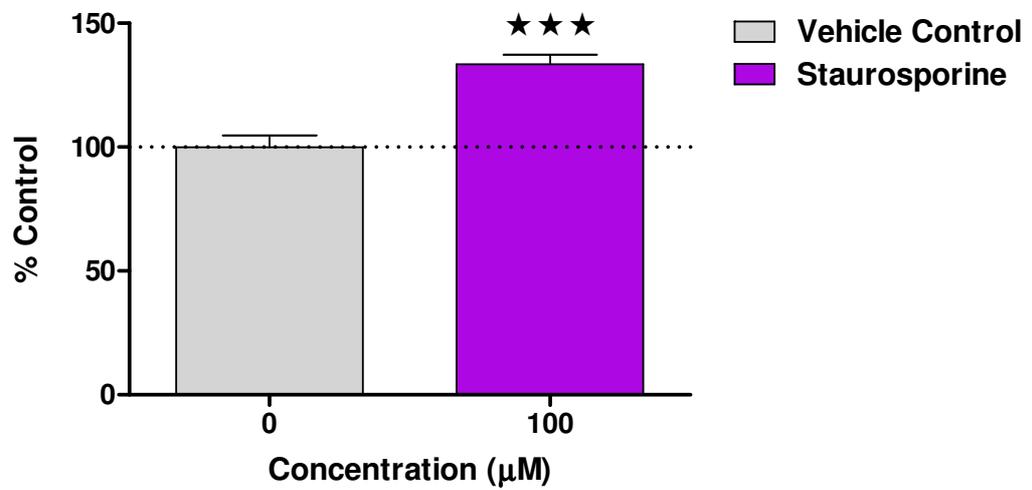


Figure 20. Caspase-3 activity observed in HepG2 cells exposed for 6 h to the positive control, Staurosporine, compared to caspase-3 activity observed in vehicle control cells. A Significant difference from the vehicle control is indicated by *******, p values < 0.001.

3.2 Effects of plant extracts on mechanistic parameters.

Cells were pre-treated with methanol extracts of BA, SC, or NAC and subjected to the battery of tests described earlier (2.4.1 -2.4.6) to determine whether they possessed a protective effect against the toxicity induced by PCP and its metabolites.

3.2.1 Cell viability

Survival curves for HepG2 cells exposed to PCP, after pre-treatment with NAC, SC and BA is depicted in Figures 21.

Cells treated with BA prior to PCP exposure showed the greatest increase in IC_{50} , where a 5-fold increase in this value was detected. This was followed by cells pre-treated with SC where the IC_{50} value increased almost 4-fold and finally by NAC which had a less than 2-fold increase in IC_{50} value (Table 3).

Cell viability in HepG2 cells pre-treated with NAC did not differ significantly from cells exposed to PCP alone (Figure 22A). Pre-treatment with SC and BA caused significant ($p < 0.05$) increases in viability of HepG2 cells when exposed to 50 -150 μ M PCP, compared to cells exposed to PCP alone (Figure 22 B + C). Increases and decreases in cell viability in cells pre-treated with NAC, BA or SC prior to PCP exposure compared to cells exposed to PCP alone, are presented in Table 3.

Table 3. Comparison of IC₅₀ values and changes in cell viability of cells pre-treated with NAC, SC and BA prior to PCP exposure compared to cells only exposed to PCP.

PCP	No pre-treatment	Pre-treatment with NAC	Pre-treatment with SC	Pre-treatment with BA
5 µM	88	- 4 .00	+ 10.83	+ 8.17
10 µM	78.89	+ 7.11	+ 20.28	+ 19.44
50 µM	52.61	+ 6.89	+ 29.39	+ 27.72
100 µM	44.44	+ 8.89	+ 24.56	+ 30.06
150 µM	37.33	+ 6.00	+ 27.34	+ 30.17
IC ₅₀	68.1 µM	104.5 µM	250.6 µM	354.8 µM

* Increases and decreases in viability are indicated by + and – values, respectively.

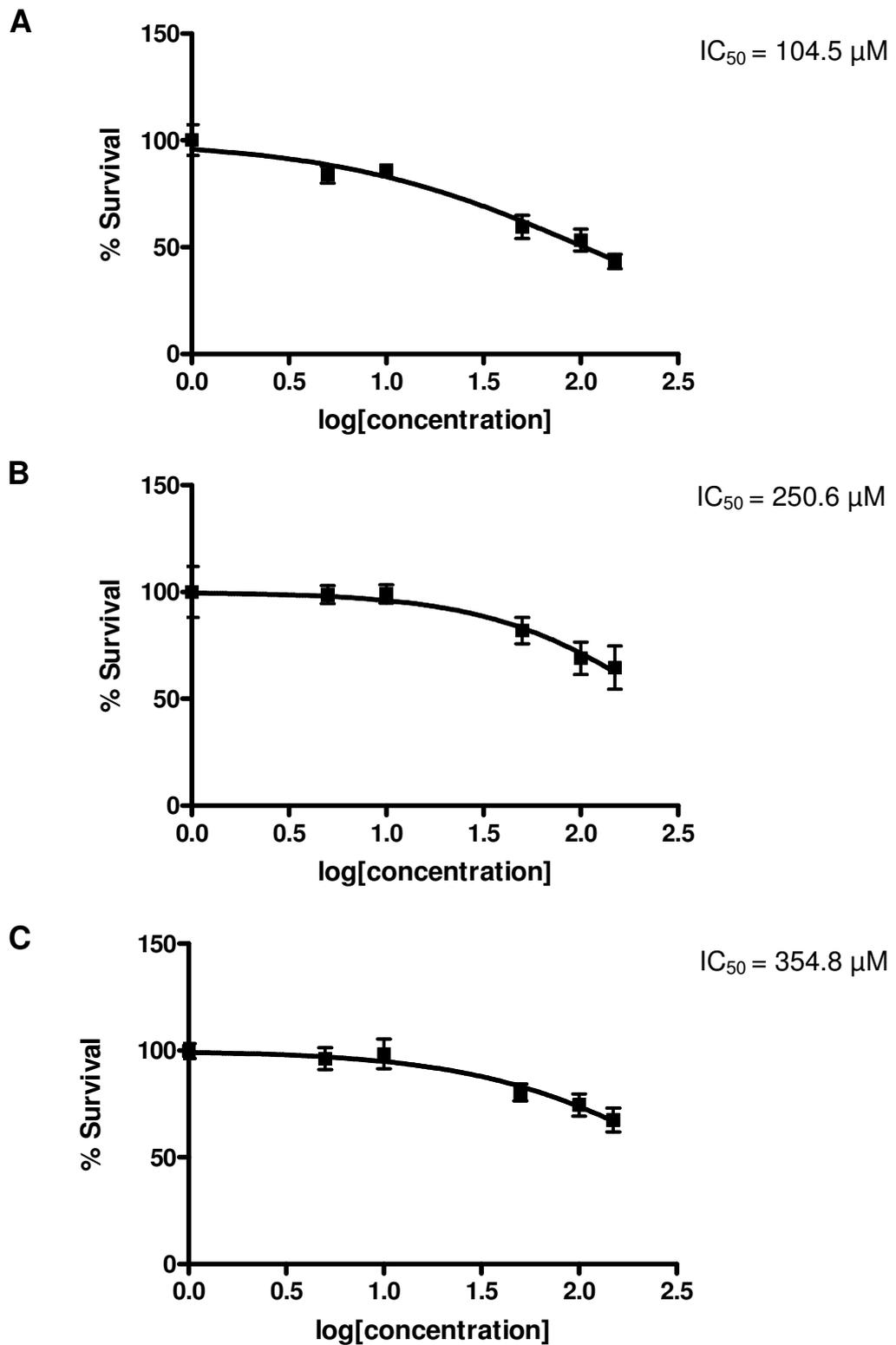


Figure 21. Survival curves of HepG2 cells exposed to PCP after pre-treatment with (A) NAC (B) SC or (C) BA.

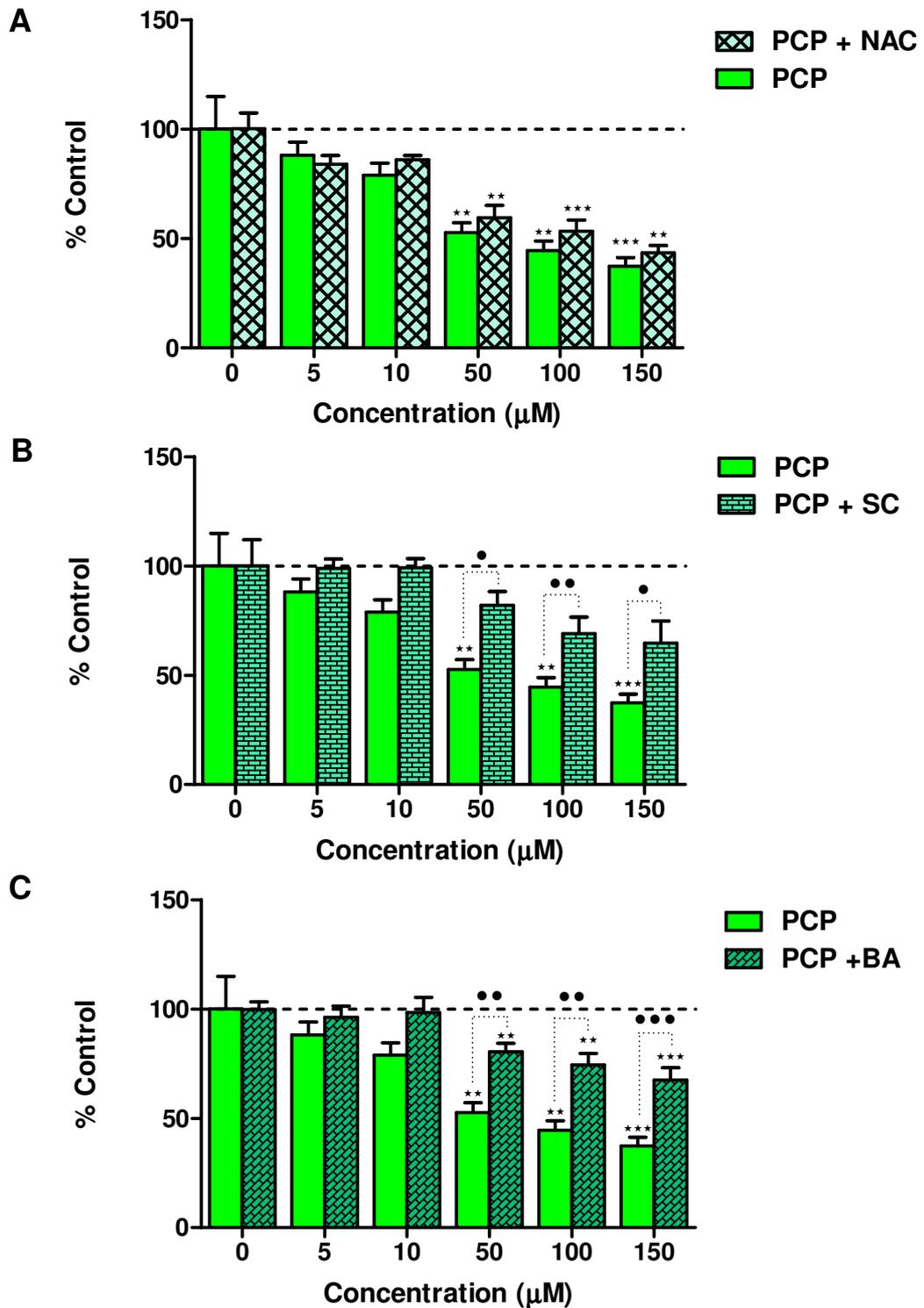


Figure 22. Cell viability in HepG2 cells exposed to PCP alone compared to cell viability in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control are indicated by ** and *** representing p values < 0.005 and 0.0001, respectively. Significant difference between groups exposed to pesticides directly and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• representing p values < 0.05, 0.01, 0.001, respectively.

Survival curves for HepG2 cells exposed to TCHQ, after pre-treatment with NAC, SC and BA are depicted in Figure 23.

A different trend was observed in cells exposed to TCHQ after pre-treatment with NAC and plant extracts, where pre-treatment with SC caused the greatest increase in the IC_{50} value followed by BA and then NAC (Table 4). These increases were far less extensive than those observed in cells exposed to PCP.

Cells pre-treated with NAC prior to TCHQ exposure showed decreases in cell viability compared to cells exposed to TCHQ alone, however the only significant ($p < 0.05$) decrease was at 100 μM (Figure 24A). Although small increases in viability were observed in cells pre-treated with SC before TCHQ exposure compared to cells exposed to TCHQ alone, BA seemed to be the only compound which induced cellular proliferation (Figure 24B and C). The only significant ($p < 0.05$) increase in viability in cells pre-treated with SC occurred when exposed to 10 μM TCHQ. Increases and decreases in cell viability in cells pre-treated with NAC, BA or SC prior to TCHQ exposure compared to cells exposed to TCHQ alone, are presented in Table 4.

Table 4. Comparison of IC₅₀ values and changes in cell viability of cells pre-treated with NAC, SC and BA prior to TCHQ exposure compared to cells only exposed to TCHQ.

TCHQ	No pre-treatment	Pre-treatment with NAC	Pre-treatment with SC	Pre-treatment with BA
5 µM	95	- 9	+ 10.7	+ 17.5
10 µM	93.33	- 8	+ 10.17	+ 20.37
50 µM	93.67	- 16.5	+ 3.66	+ 10.63
100 µM	79.17	- 30.34	- 3.34	+ 4.66
150 µM	45.83	- 11.33	+ 4.84	+ 0.17
IC ₅₀	144.0 µM	99.4 µM	150.6 µM	144.6 µM

* Increases and decreases in viability are indicated by + and – values, respectively.

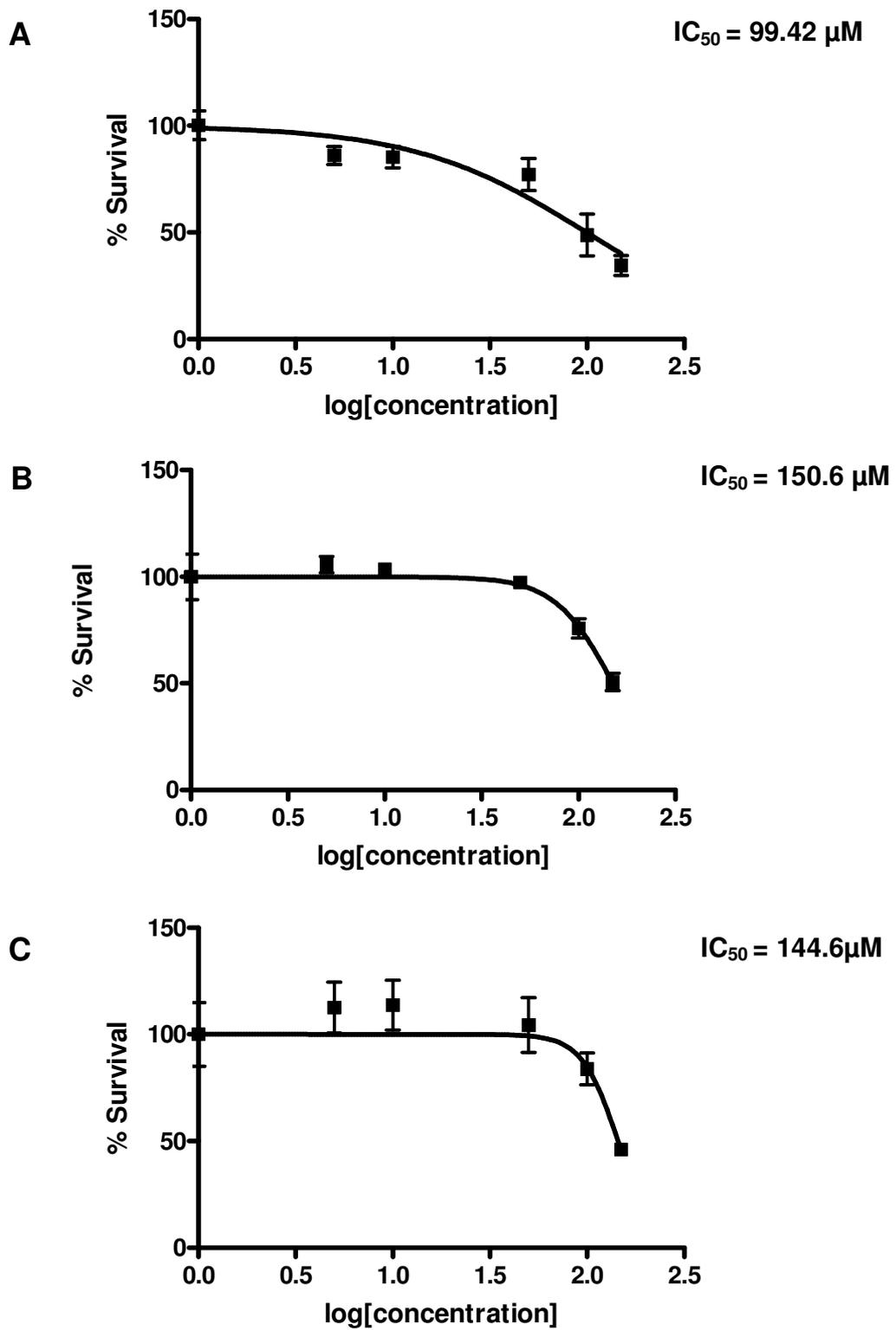


Figure 23. Survival curves of HepG2 cells exposed to TCHQ after pre-treatment with (A) NAC (B) SC or (C) BA.

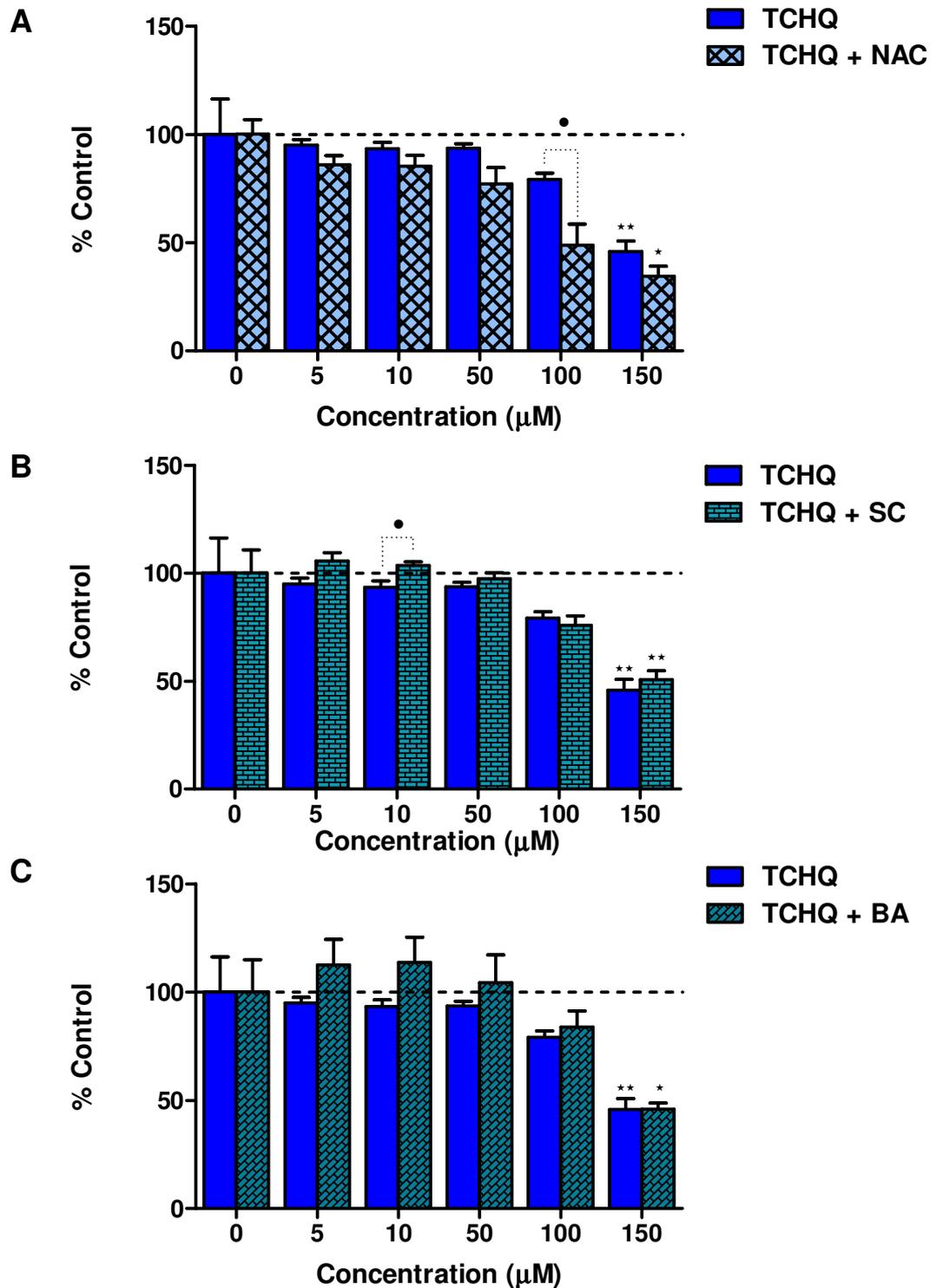


Figure 24. Cell viability in HepG2 cells exposed to TCHQ alone compared to cell viability in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCHQ exposure. Significant difference from the vehicle control is indicated by * and ** representing p values < 0.05 and 0.001, respectively. Significant differences between groups exposed to pesticides directly and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, p value < 0.05.

Survival curves for HepG2 cells exposed to TCBQ, after pre-treatment with NAC, SC and BA is depicted in Figure 25.

A similar trend to TCHQ was seen in cells exposed to TCBQ where pre-treatment with SC once again caused the greatest increase in the IC₅₀ value followed by BA and then NAC (Table 5). These increases were once again far less extensive than those observed in cells exposed to PCP.

Although not significant, cells pre-treated with NAC prior to TCBQ exposure showed a decrease in cell viability at 50 and 100 μ M compared to cells exposed to TCBQ alone (Figure 26A). An increase in cell viability was seen when cells were pre-treated with NAC and exposed to 150 μ M TCBQ compared to cells exposed to TCBQ alone. This increase was not significant (Figure 26A). A significant ($p < 0.001$) increase in cell viability was only observed in cells treated with SC prior to exposure to 150 μ M TCBQ compared to cells treated with TCBQ alone (Figure 26B). Increases in viability were observed in cells pre-treated with BA before TCBQ exposure compared to cells exposed to TCBQ alone. BA once again was the only compound which produced slight cellular proliferation in cells exposed to the lowest concentration of TCBQ. Increases and decreases in cell viability in cells pre-treated with NAC, BA or SC prior to TCBQ exposure compared to cells exposed to TCBQ alone, is presented in Table 5.

Table 5. Comparison of IC₅₀ values and changes in cell viability of cells pre-treated with NAC, SC and BA prior to TCBQ exposure compared to cells only exposed to TCBQ.

TCBQ	No pre-treatment	Pre-treatment with NAC	Pre-treatment with SC	Pre-treatment with BA
5 μM	96.94	- 3.27	+ 1.89	-0.77
10 μM	95.22	+ 1.11	+ 3.95	+ 3.11
50 μM	92.83	- 21.16	-10.83	-12.5
100 μM	77.61	- 18.28	- 8.61	- 3.11
150 μM	33.11	+ 13.72	+ 31.56	+ 34.39
IC₅₀	129.4 μM	126.5 μM	182.9 μM	146.6 μM

* Increases and decreases in viability are indicated by + and – values, respectively.

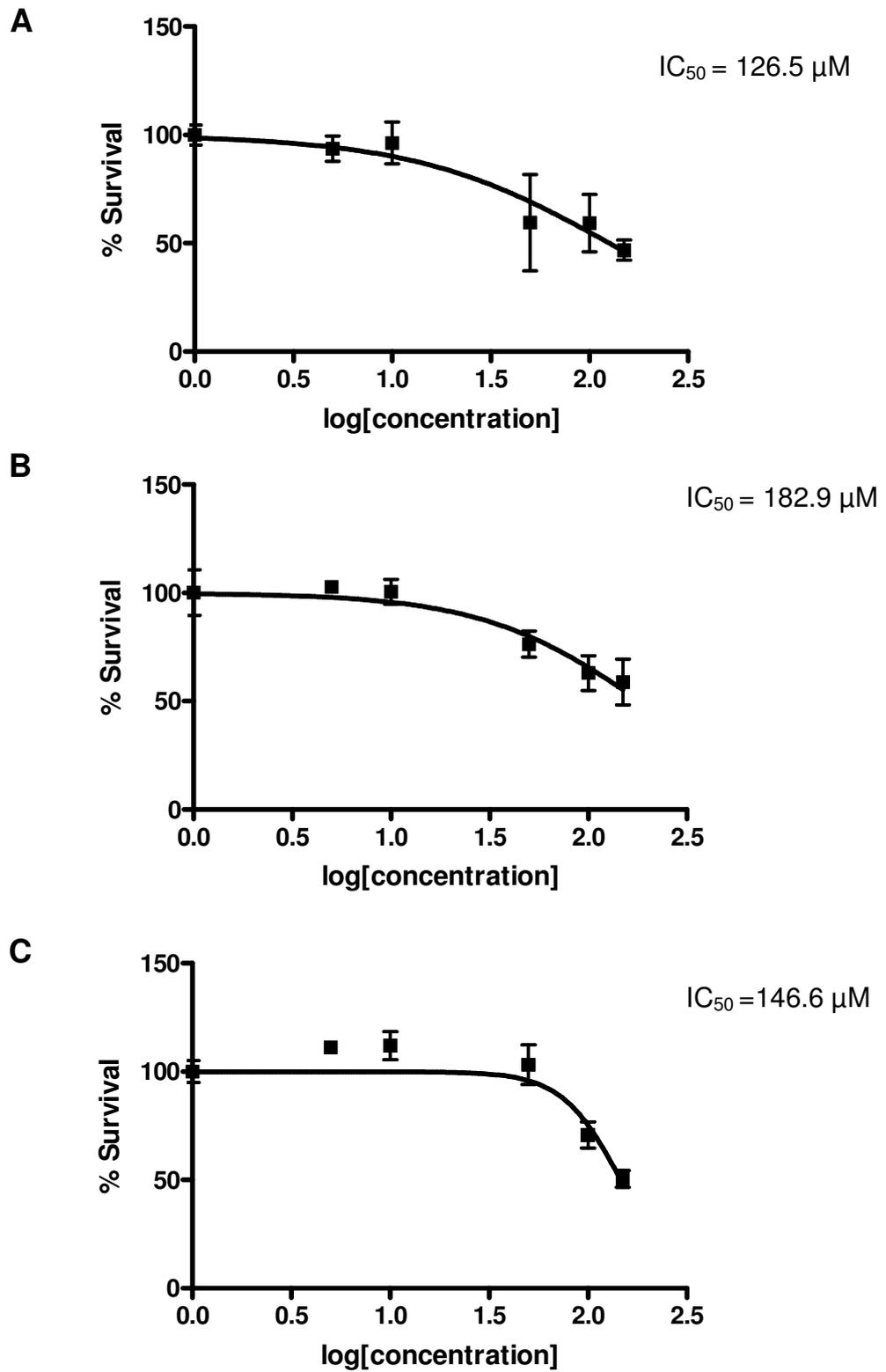


Figure 25. Survival curves of HepG2 cells exposed to concentrations of TCBQ after pre-treatment with (A) NAC (B) SC or (C) BA.

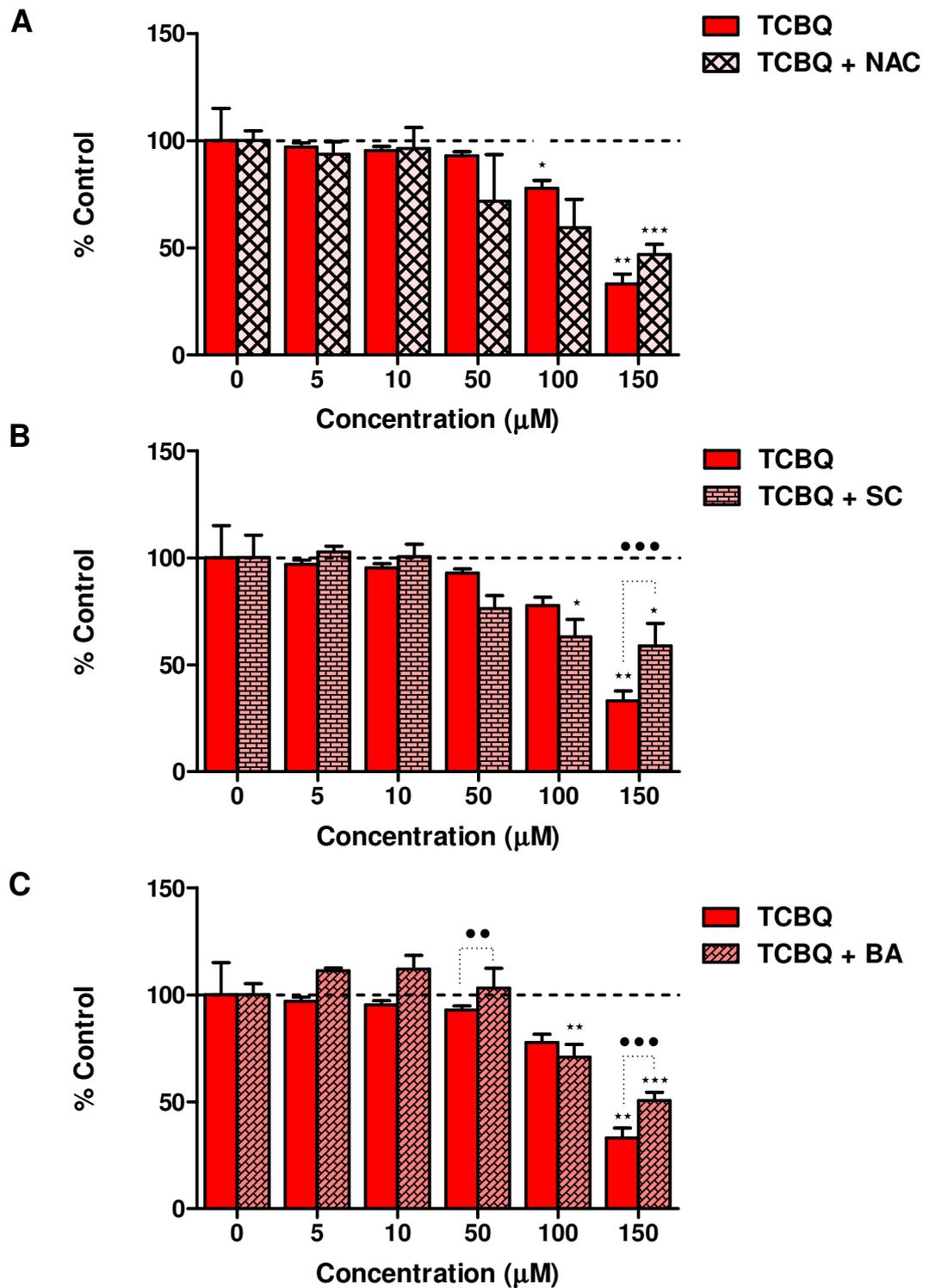


Figure 26. Cell viability in HepG2 cells exposed to TCBQ alone compared to cell viability in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control are indicated by *, ** and *** representing p values < 0.05, 0.01 and 0.001, respectively. Significant difference between groups exposed to pesticides directly and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •• and ••• representing p values < 0.01 and 0.001, respectively.

3.2.2 CYP 1A1 activity

A decrease in CYP1A1 activity was seen when cells were pre-treated with NAC, SC or BA prior to PCP exposure, compared to cells exposed to PCP alone (Figure 27). These decreases were significant ($p < 0.05$) with the exception of the decrease observed in cells treated with NAC prior to exposure to 5 μM PCP (Figure 27A). Pre-treatment with SC, prior to PCP exposure, led to the largest decrease in CYP1A1 activity (Figure 27B). Compared to vehicle controls, cells pre-treated with NAC resulted in significantly ($p < 0.05$) higher CYP1A1 activity when exposed to concentrations of PCP ranging from 5 to 50 μM , whilst a significant ($p < 0.05$) decrease from the vehicle control was seen when exposed to 150 μM .

Cells pre-treated with NAC showed a slight increase in CYP1A1 activity after exposure to 5 and 10 μM of TCHQ, compared to cells exposed to the metabolite alone (Figure 28A). This increase was only significant ($p < 0.01$) in cells exposed to 10 μM TCHQ. Although not significant, decreases in CYP1A1 activity was observed in cells pre-treated with NAC and exposed to 50 - 150 μM of TCHQ, compared to cells exposed to TCHQ alone. Cells pre-treated with SC before TCHQ exposure followed a similar trend as seen in cells pre-treated with NAC with a significant ($p < 0.01$) increase in CYP1A1 when exposed to 5 and 10 μM TCHQ (Figure 28B). Negligible differences in CYP1A1 activity could be seen in cells pre-treated with SC before exposure to 50, 100 and 150 μM of TCHQ. Significant ($p < 0.05$) induction of CYP1A1 activity was seen in cells pre-treated with BA prior to exposure to 5 - 100 μM of TCHQ, compared to cells only treated with the respective concentrations of TCHQ (Figure 28C). Although not significant a decrease in CYP1A1 activity was seen in BA pre-treated cells exposed to 150 μM TCHQ compared to CYP1A1 activity in cells only exposed to TCHQ.

Increases in CYP1A1 activity was observed in cells pre-treated with NAC prior to exposure to 5, 10 and 150 μM of TCBQ. This was not significant when compared to cells exposed to the respective concentrations of TCBQ alone (Figure 29 A). Significant ($p < 0.05$) decreases in CYP1A1 activity was observed in cells pre-treated with NAC

before exposure to 50 and 100 μM of TCBQ, compared to cells exposed to TCBQ alone.

Cells pre-treated with SC before TCBQ exposure showed a significant ($p < 0.01$) increase in CYP1A1 activity when exposed to 10 μM TCBQ and a significant decrease ($p < 0.05$) in CYP1A1 activity when exposed to 50 and 100 μM TCBQ, compared to cells exposed to TCBQ without pretreatment (Figure 29B). A significant ($p < 0.001$) increase in CYP1A1 activity was noted in cells pretreated with BA before exposure to 5 and 10 μM TCBQ compared to cells only treated with TCBQ (Figure 29C). A significant ($p < 0.05$) decrease in CYP1A1 activity was observed in BA pre-treated cells when exposed to 100 μM TCBQ compared to cells directly exposed to TCBQ without any pretreatment.

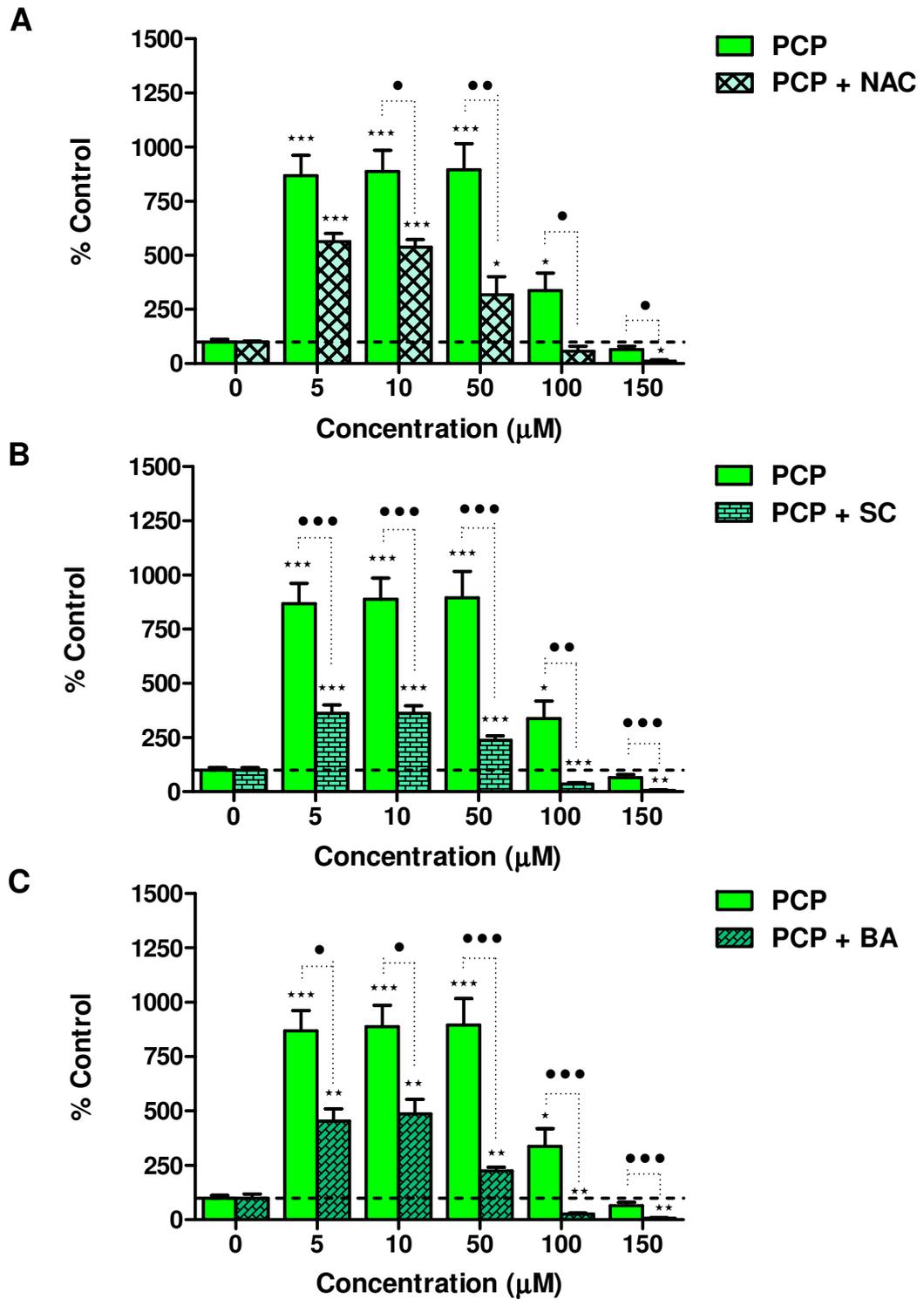


Figure 27. CYP1A1 activity observed in HepG2 cells directly exposed to PCP compared to CYP 1A1 in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control is indicated by *, ** and *** representing p values < 0.05, 0.005 and 0.0001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• representing p values < 0.05, 0.005, 0.0001, respectively.

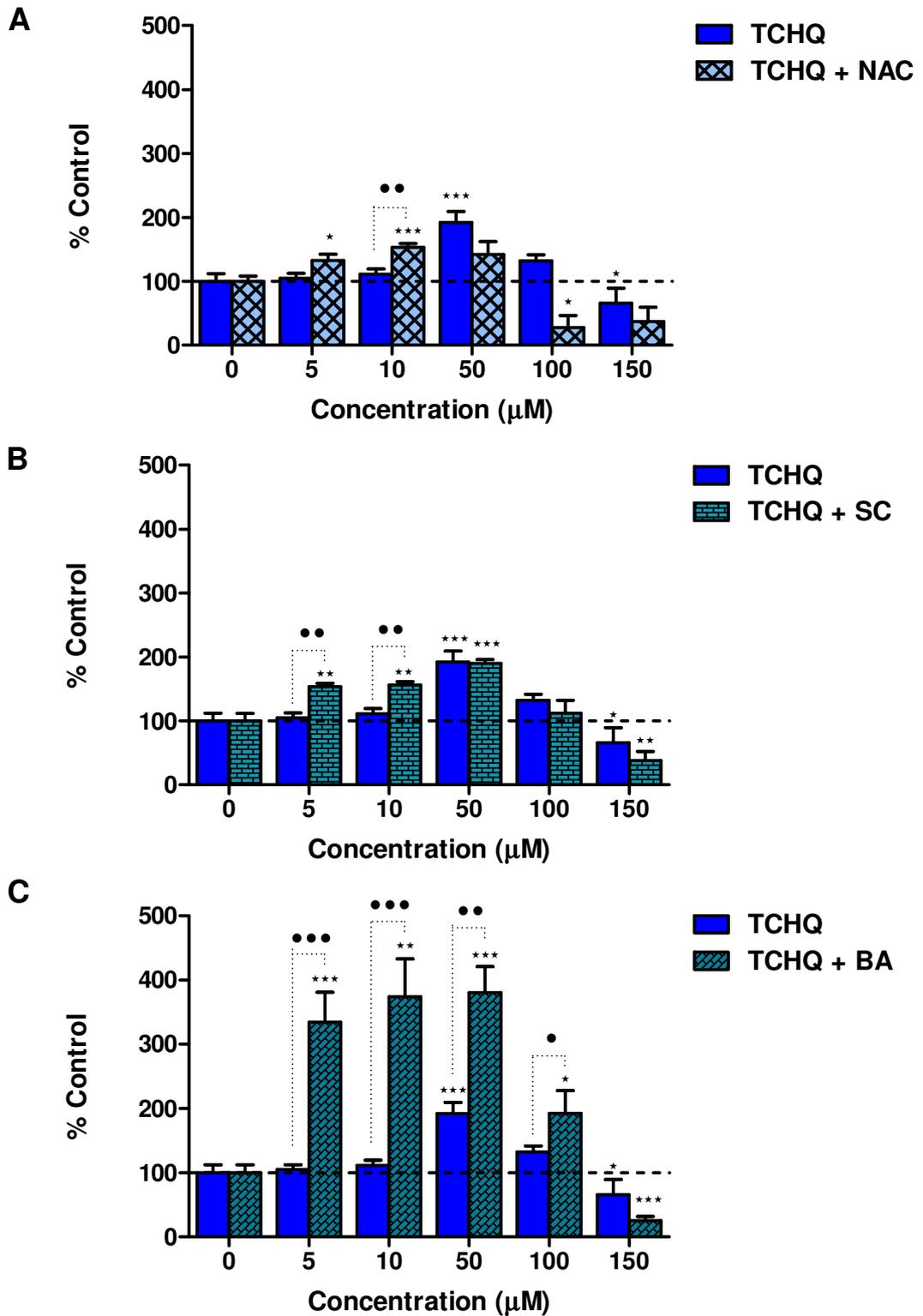


Figure 28. CYP1A1 activity observed in HepG2 cells directly exposed to TCHQ compared to CYP 1A1 in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA. Significant difference from the vehicle control is indicate by *, ** and *** representing p values < 0.05, 0.01 and 0.001, respectively. Significant difference between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• representing p values < 0.05, 0.01, 0.001, respectively.

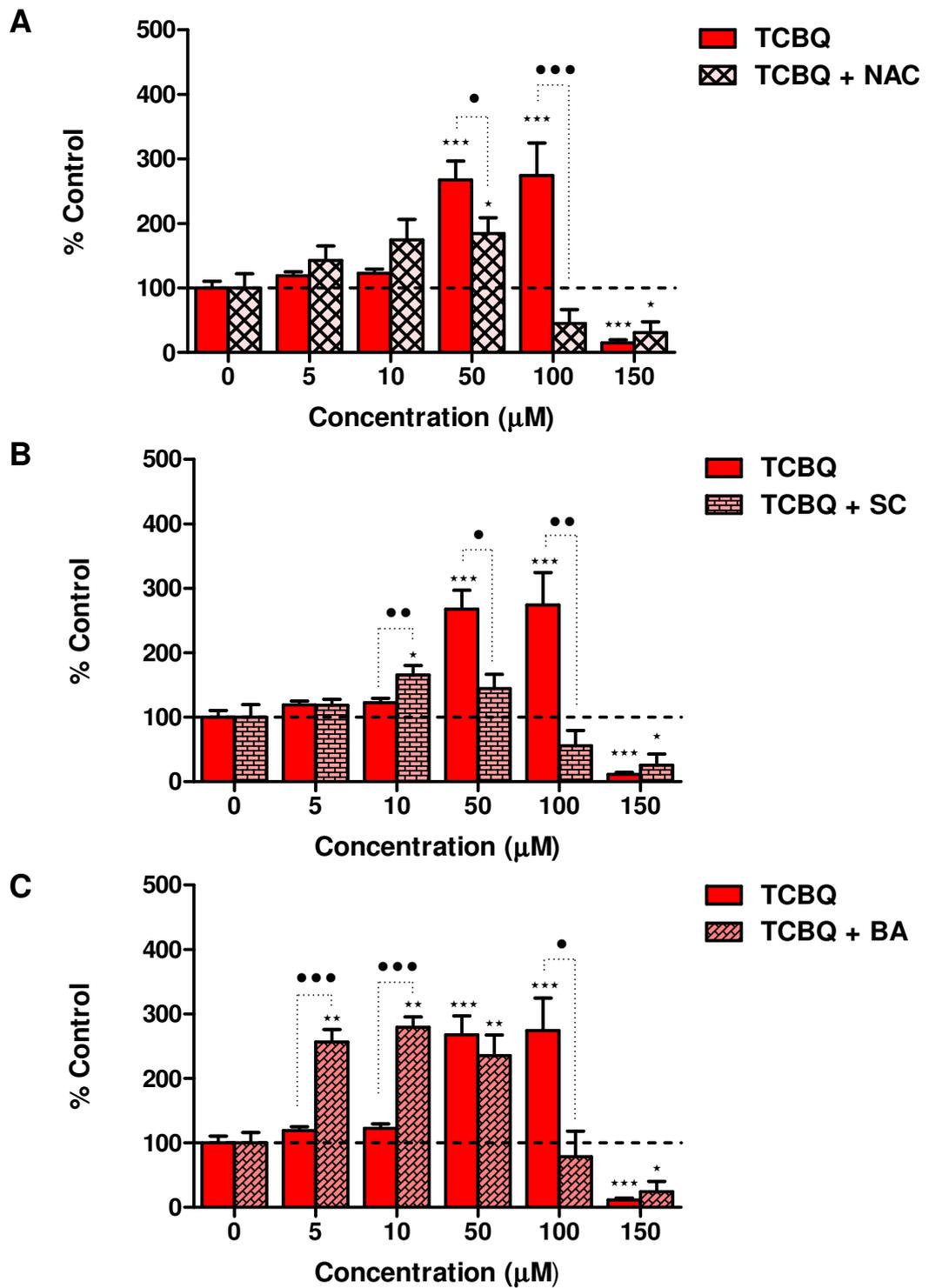


Figure 29. CYP1A1 activity observed in HepG2 cells directly exposed to TCBQ compared to CYP 1A1 in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control are indicated by *, ** and *** representing p values < 0.05, 0.01 and 0.001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by •, ••, ••• representing p values < 0.05, 0.01, 0.001, respectively.

3.2.3 Reactive oxygen species generation

Treatment of HepG2 cells with NAC prior to PCP exposure inhibited the decrease in intracellular ROS caused by PCP alone at concentrations of 10 and 50 μM . An increase in ROS generation was observed when cells were exposed to 100 and 150 μM PCP however, this increase was only significant ($p < 0.05$) at 100 μM (Figure 30A). The increase seemed to follow a dose response which reached a plateau between 100 and 150 μM . No significant difference was seen in the amount of ROS generation in cells pre-treated with NAC prior to PCP exposure compared to the ROS generation in the vehicle control cells. Pre-treatment with SC caused a significant ($p < 0.05$) decrease in intra-cellular ROS in cells exposed to PCP at all test concentrations compared to cells exposed directly to PCP (Figure 30B). Due to large standard deviations in the data no significant difference was seen in the amount of ROS in cells pre-treated with SC prior to PCP exposure compared to vehicle controls. Pre-treatment with BA also caused decreases in ROS caused by exposure to PCP at all test concentrations (Figure 30C). These decreases, however were only significant ($p < 0.05$) when cells were exposed to 5, 10 and 150 μM PCP.

Pre-treatment of HepG2 cells with NAC, as well as SC and BA, inhibited ROS generation due to TCHQ exposure at all concentrations tested (Figure 31). The decreases were significant ($p < 0.05$) for cells pre-treated with NAC and BA when exposed to 10 μM TCHQ and for cells pre-treated with SC when exposed to 5 and 10 μM TCHQ.

As in cells exposed to TCHQ, cells pre-treated with NAC as well as SC and BA inhibited ROS generation caused by exposure to TCBQ (Figure 32). These decreases were significant ($p < 0.05$) in cells pre-treated with NAC when exposed to 5 and 10 μM TCBQ and in cells pre-treated with SC when exposed to all test concentrations of TCBQ. The decreases in ROS generation in cells treated with BA prior to TCBQ exposure, however, was not significant at any concentration. Significant ($p < 0.05$) increases in ROS generation compared to vehicle control were evident in cells pre-treated with NAC, BA and SC prior to exposure to all concentrations of TCBQ.

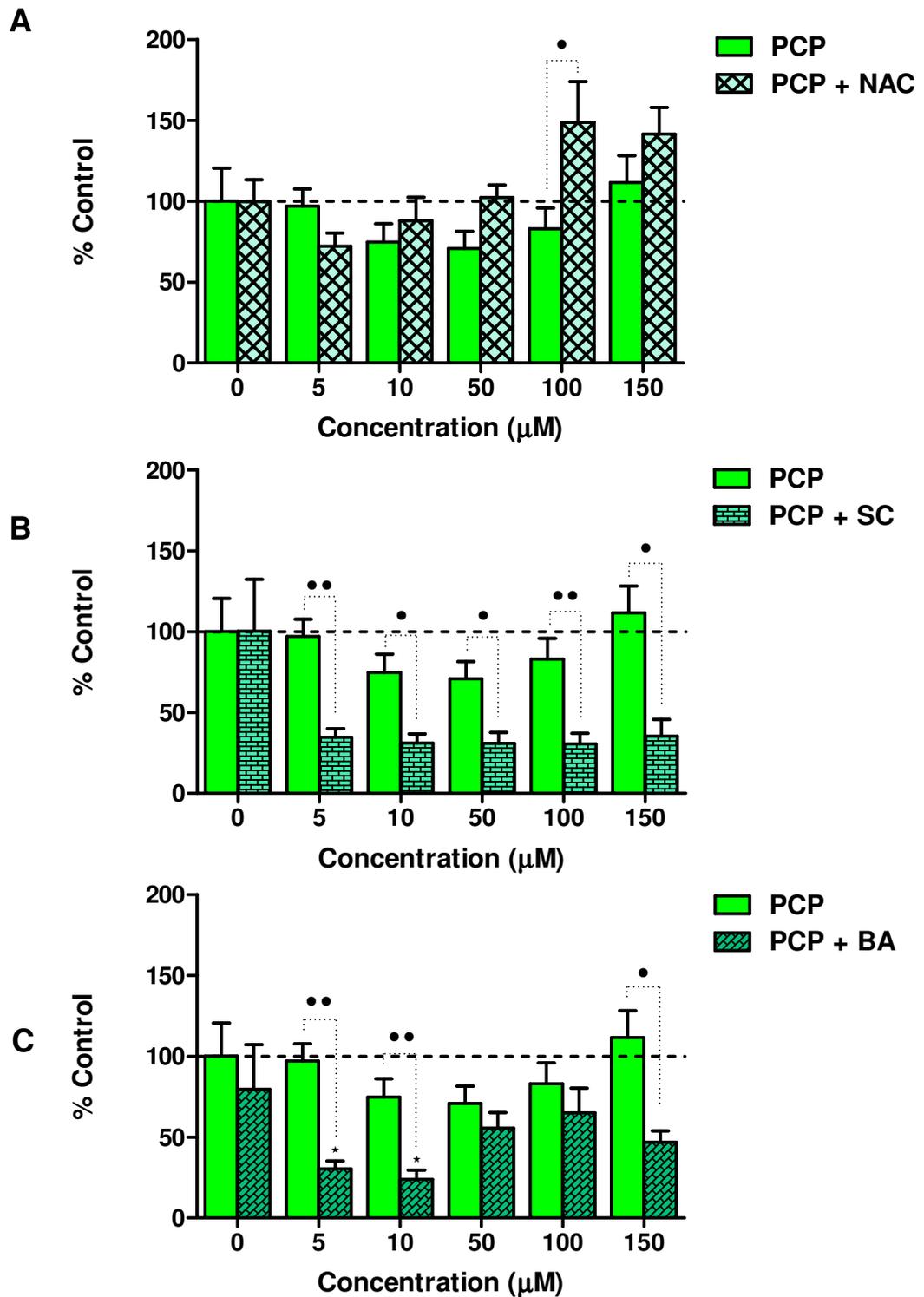


Figure 30. ROS generation observed in HepG2 cells directly exposed to PCP compared to ROS generation in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control are indicated by *, p values < 0.05. Significant differences between groups exposed directly to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by • and •• representing p values < 0.05 and 0.01, respectively.

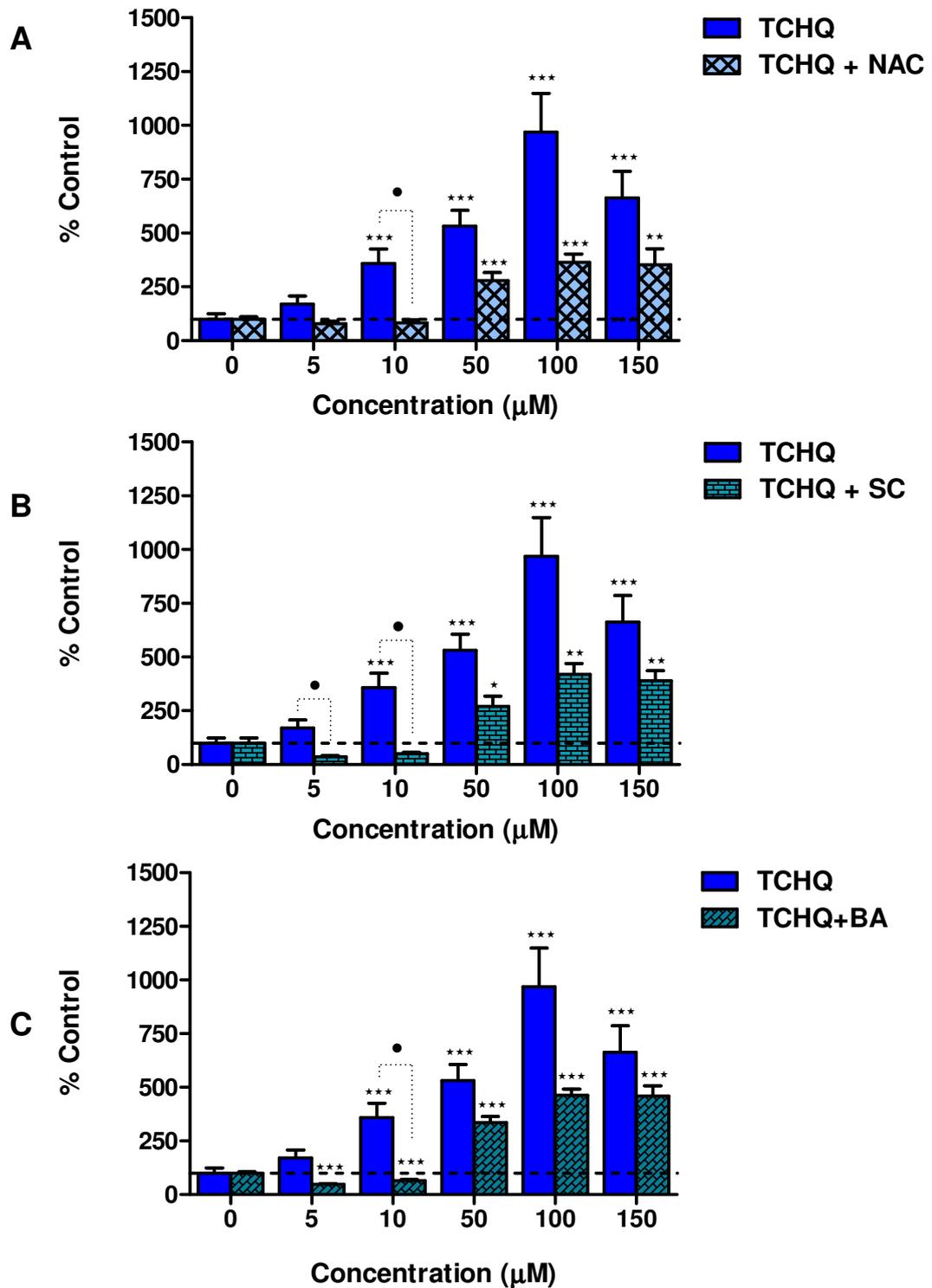


Figure 31. ROS generation observed in HepG2 cells directly exposed to TCHQ compared to ROS generation in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCHQ exposure. * Significant differences from the vehicle control are indicated by *, ** and *** representing p values < 0.05, 0.01 and 0.001, respectively. Significant differences between groups directly exposed to pesticides, and groups first pre-treated with NAC or plant extracts for each concentration are indicated by •, p values < 0.05.

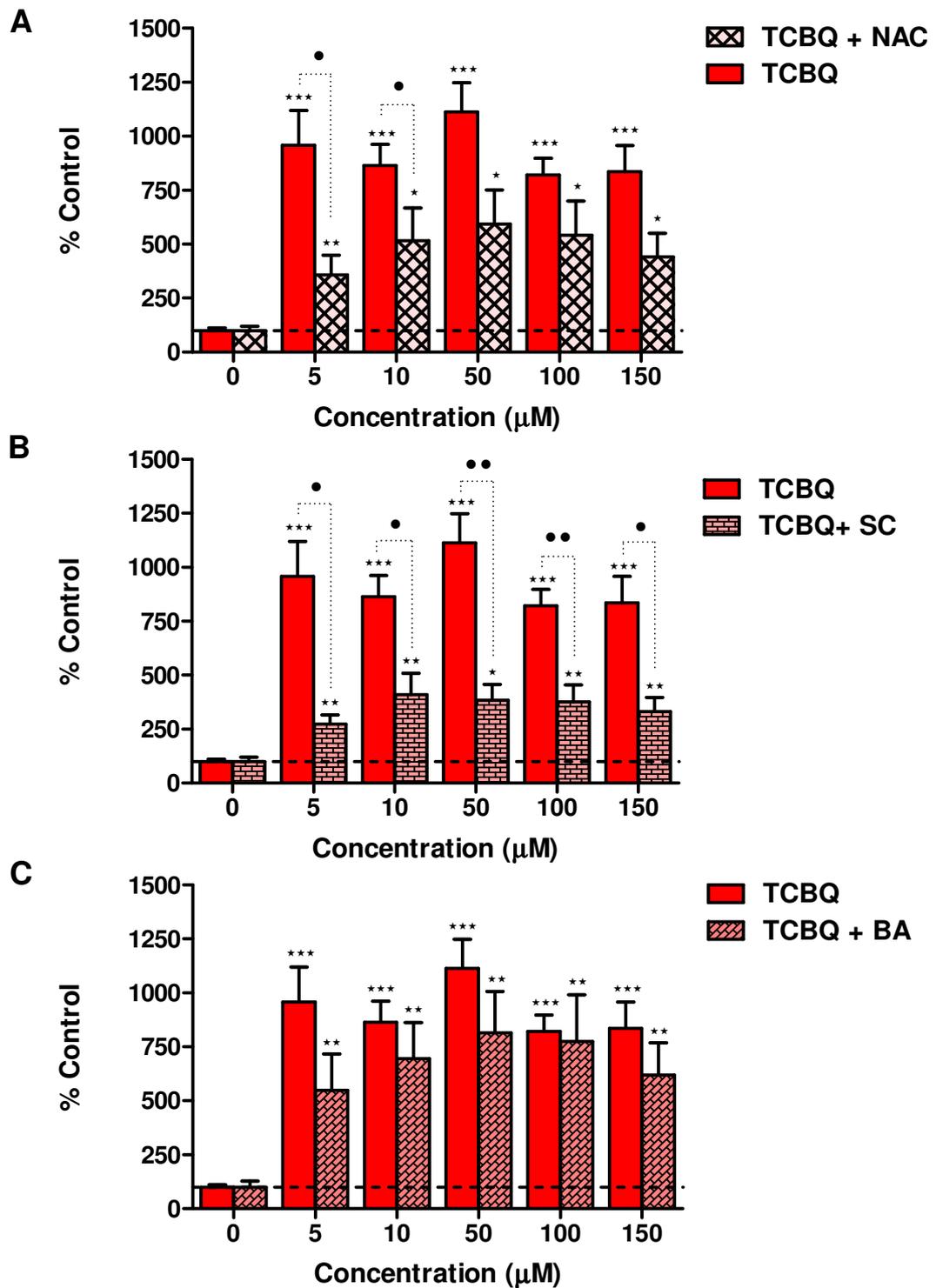


Figure 32. ROS generation observed in HepG2 cells directly exposed to TCBQ compared to ROS generation in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control are indicated by *, ** and *** representing p values < 0.05, 0.005 and 0.0001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by • and •• representing p values < 0.05 and 0.01, respectively.

3.2.4 Mitochondrial membrane potential

No significant change in MMP was observed in HepG2 cells pre-treated with NAC prior to PCP exposure (Figure 33A). More extensive mitochondrial depolarization occurred in cells pre-treated with both SC and BA when exposed to all test concentrations of PCP (Figure 33B+C). However no dose-response was observed for SC and BA treated cells indicating a maximal response.

In cells pre-treated with NAC, increases in MMP could be seen when exposed to 5 and 10 μM of TCHQ, compared to cells exposed to the respective concentrations of TCHQ alone (Figure 34A). This increase was only significant ($p < 0.01$) at 5 μM TCHQ. Significant ($p < 0.001$) decreases in MMP compared to the vehicle control were noted in cells pre-treated with NAC and then exposed to 50 -150 μM concentrations of TCHQ. A significant ($p < 0.05$) decrease in MMP occurred in cells pre-treated with SC when exposed to 5 μM TCHQ compared to cells exposed to TCHQ alone (Figure 34B). Significant ($p < 0.05$) decreases in MMP compared to the vehicle control were noted in cells pre-treated with SC and then exposed to all test concentrations of TCHQ. Treatment of cells with BA prior to TCHQ exposure appeared to have negligible effects on MMP (Figure 34C).

Pre-treatment with NAC, SC or BA before exposure to TCBQ caused even more extensive mitochondrial depolarization than was observed in cells only exposed to TCBQ without pre-treatment (Figure 35). This exacerbation of depolarization was significant ($p < 0.01$) when cells were exposed to all test concentrations except 50 μM TCBQ for both cells pre-treated with NAC and BA and significant at all test concentrations of TCBQ for SC treated cells. Significant ($p < 0.001$) decreases in MMP compared to vehicle control could be seen in cells pre-treated with NAC, SC and BA prior to exposure to all concentrations of TCBQ. Again however, no dose-response was observed for SC and BA treated cells indicating a maximal response.

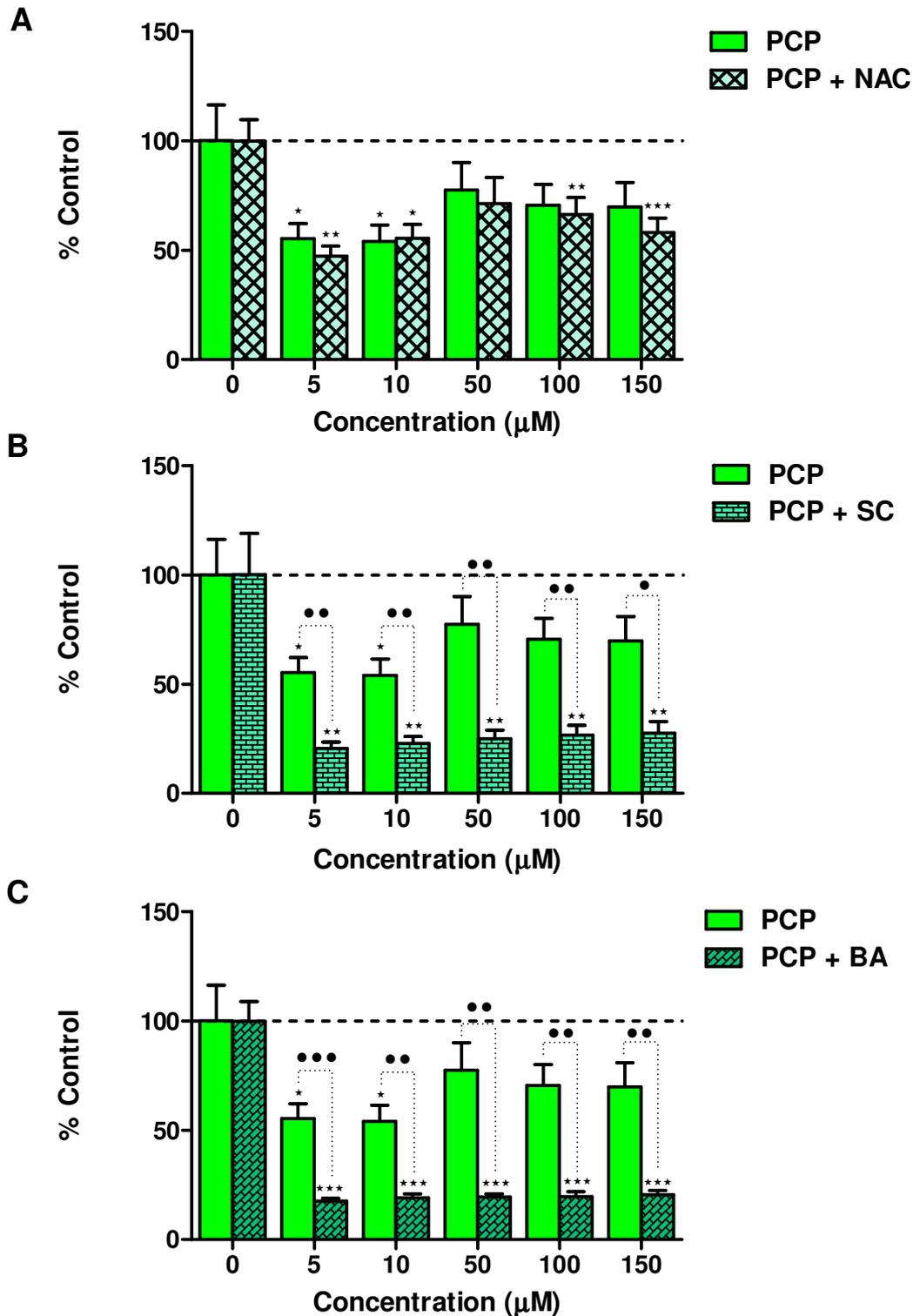


Figure 33. MMP observed in HepG2 cells directly exposed to PCP compared to MMP in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control are indicated by *, ** and *** representing p values <0.05, 0.01 and 0.001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• representing p values < 0.05, 0.01, 0.001, respectively.

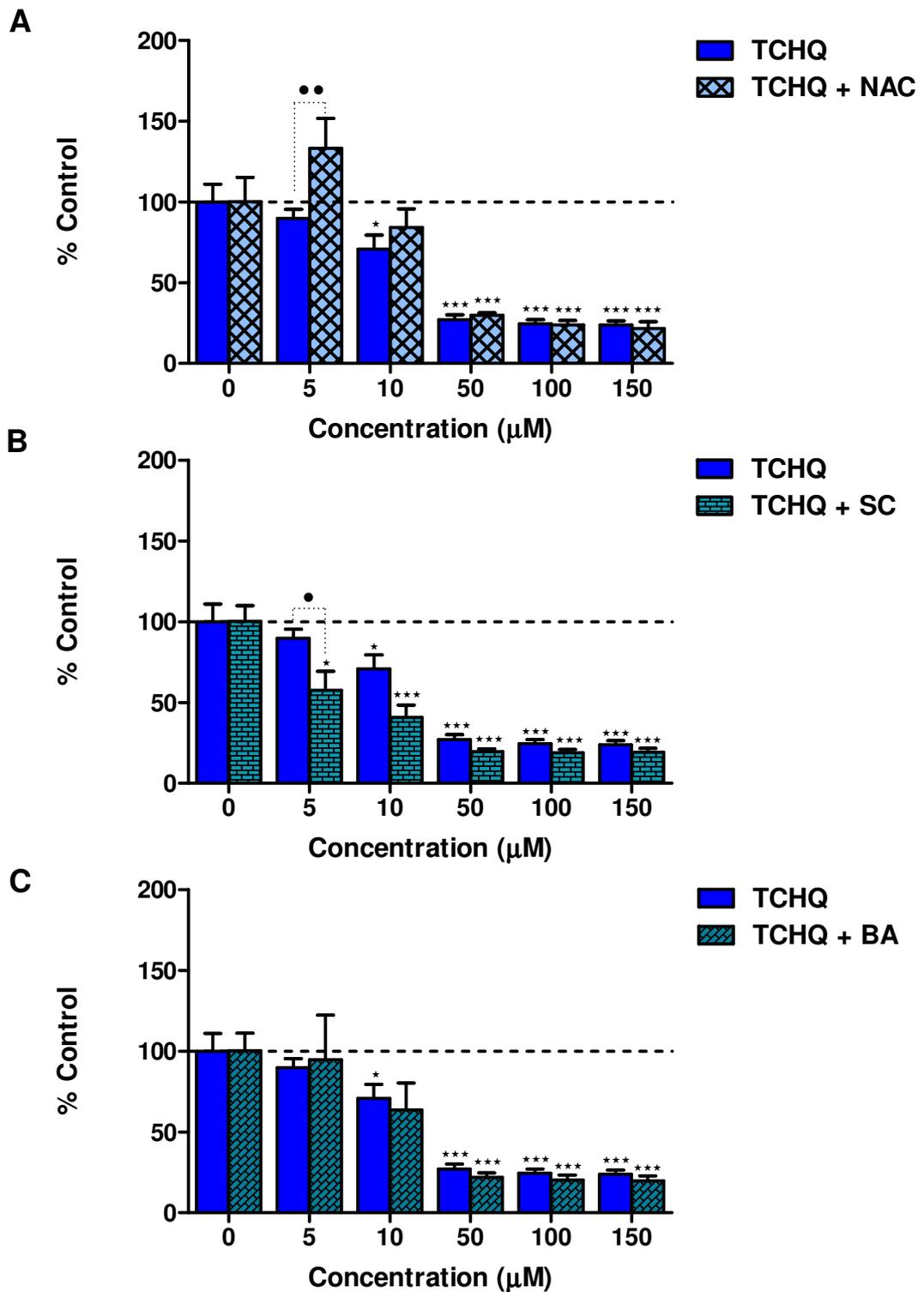


Figure 34. MMP observed in HepG2 cells exposed directly to TCHQ compared to MMP in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA to TCHQ exposure. Significant differences from the vehicle control are indicated by * and *** representing p values < 0.05 and 0.001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by • and •• representing p values < 0.05 and 0.01, respectively.

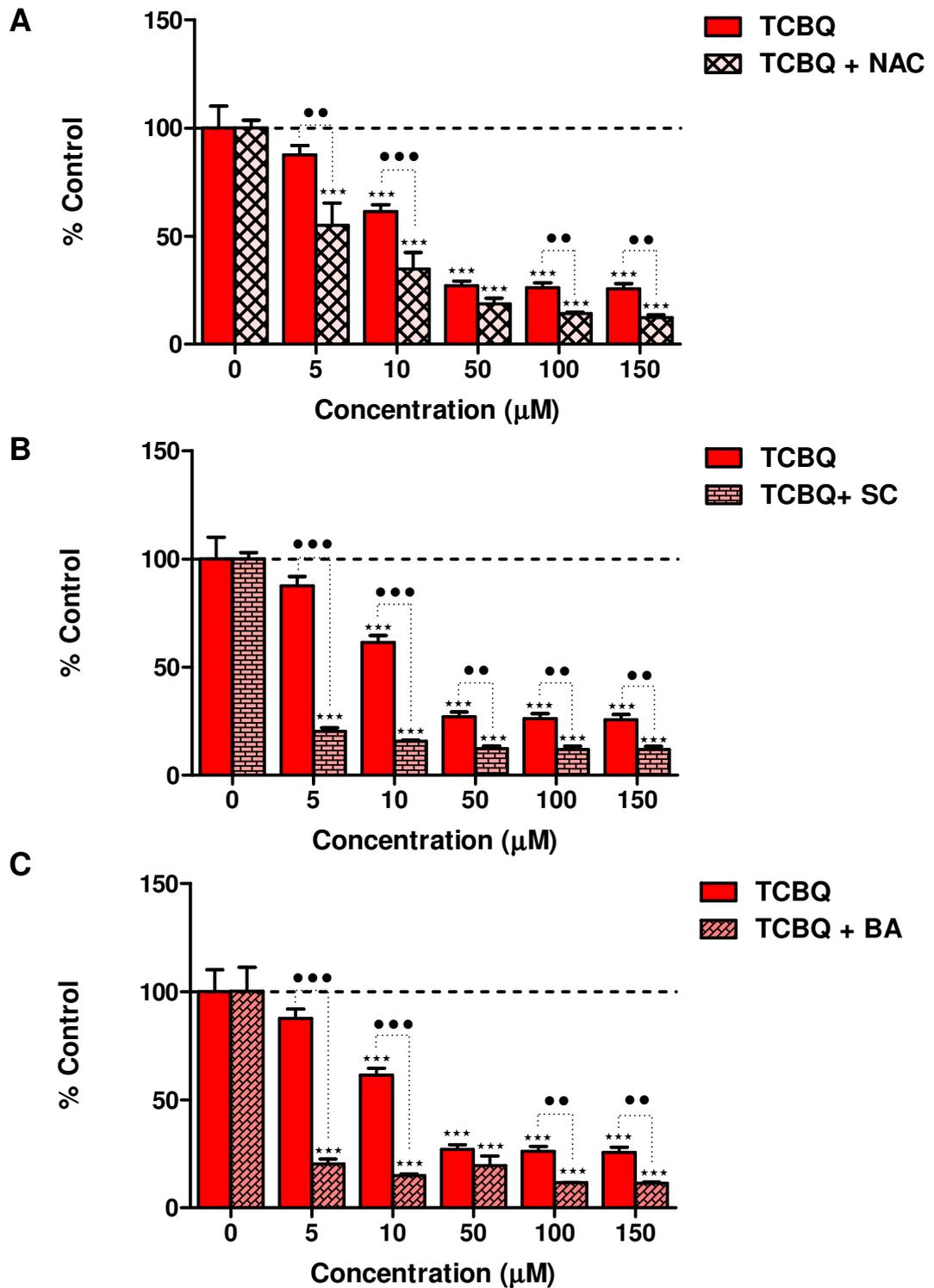


Figure 35. MMP observed in HepG2 cells directly exposed to TCBQ compared to MMP in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control are indicated by ***, p value < 0.001. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •• and ••• representing p values < 0.01 and 0.001, respectively.

3.2.5 Necrosis

Pre-treatment with NAC produced mostly negligible effects on necrosis (Figure 36A). A significant ($p < 0.05$) decrease in PI fluorescence could only be observed when cells were exposed to 5 μM PCP after pre-treatment with NAC compared to cells exposed to PCP alone. There was a significant ($p < 0.05$) decrease in PI fluorescence compared to the vehicle control in cells pre-treated with NAC when exposed to 150 μM PCP. An increase in necrosis was observed when cells were pre-treated with SC and then exposed to all concentrations of PCP (Figure 36B). These increases were significant ($p < 0.01$) at 5, 50, 100 and 150 μM of PCP. Pre-treatment with BA caused a significant ($p < 0.05$) dose-dependent increase in necrosis in cells exposed to 100 and 150 μM PCP compared to cells exposed to 100 and 150 μM PCP without pre-treatment (Figure 36C). A significant ($p < 0.05$) increase in necrosis from the vehicle control was noted in cells pre-treated with SC, when exposed to 5 and 150 μM PCP. No significant difference to vehicle control was observed in cells pre-treated with BA.

NAC caused a stabilizing effect in cells exposed to TCHQ where a significant ($p < 0.05$) decrease in PI fluorescence could be observed in cells exposed to 10 and 50 μM TCHQ after pre-treatment with NAC whilst a significant ($p < 0.05$) increase in fluorescence could be seen in cells exposed to 150 μM of TCHQ (Figure 37A). Cells exposed to TCHQ after SC pre-treatment showed significant ($p < 0.001$), dose-dependent increases in necrosis when exposed to all test concentrations of TCHQ compared to cells exposed to TCHQ alone (Figure 37B). A Significant ($p < 0.05$) increase in necrosis compared to the vehicle control was evident in cells pre-treated with SC prior to TCHQ exposure at all test concentrations. Pre-treatment with BA also caused dose-dependent increases in necrosis in cells exposed to TCHQ, compared to cells only exposed to TCHQ without pre-treatment (Figure 37 C). These increases were significant ($p < 0.05$) at 5, 10, 100 and 150 μM of TCHQ. Significant ($p < 0.05$) increases in fluorescence in cells pre-treated with BA compared to vehicle control were seen after exposure to 10, 50, 100 and 150 μM concentrations of TCHQ.

Negligible effects could be seen on necrosis in cells treated with NAC prior to TCBQ exposure (Figure 38A). As with TCHQ, pre-treatment with SC caused significant

($p < 0.01$) increases in necrosis in cells exposed to all test concentrations of TCBQ (Figure 38B). Significant ($p < 0.05$) increases in necrosis compared to the vehicle control occurred in cells pre-treated with SC prior to exposure to all test concentrations of TCBQ (Figure 38B). Pre-treatment with BA caused dose-dependent increases in necrosis when exposed to higher concentrations of TCBQ (Figure 38C). These increases were significant ($p < 0.01$) at 100 and 150 μM of TCBQ. Significant ($p < 0.01$) increases in necrosis compared to the vehicle control was evident in cells pre-treated with BA prior to exposure to 150 μM of TCBQ alone.

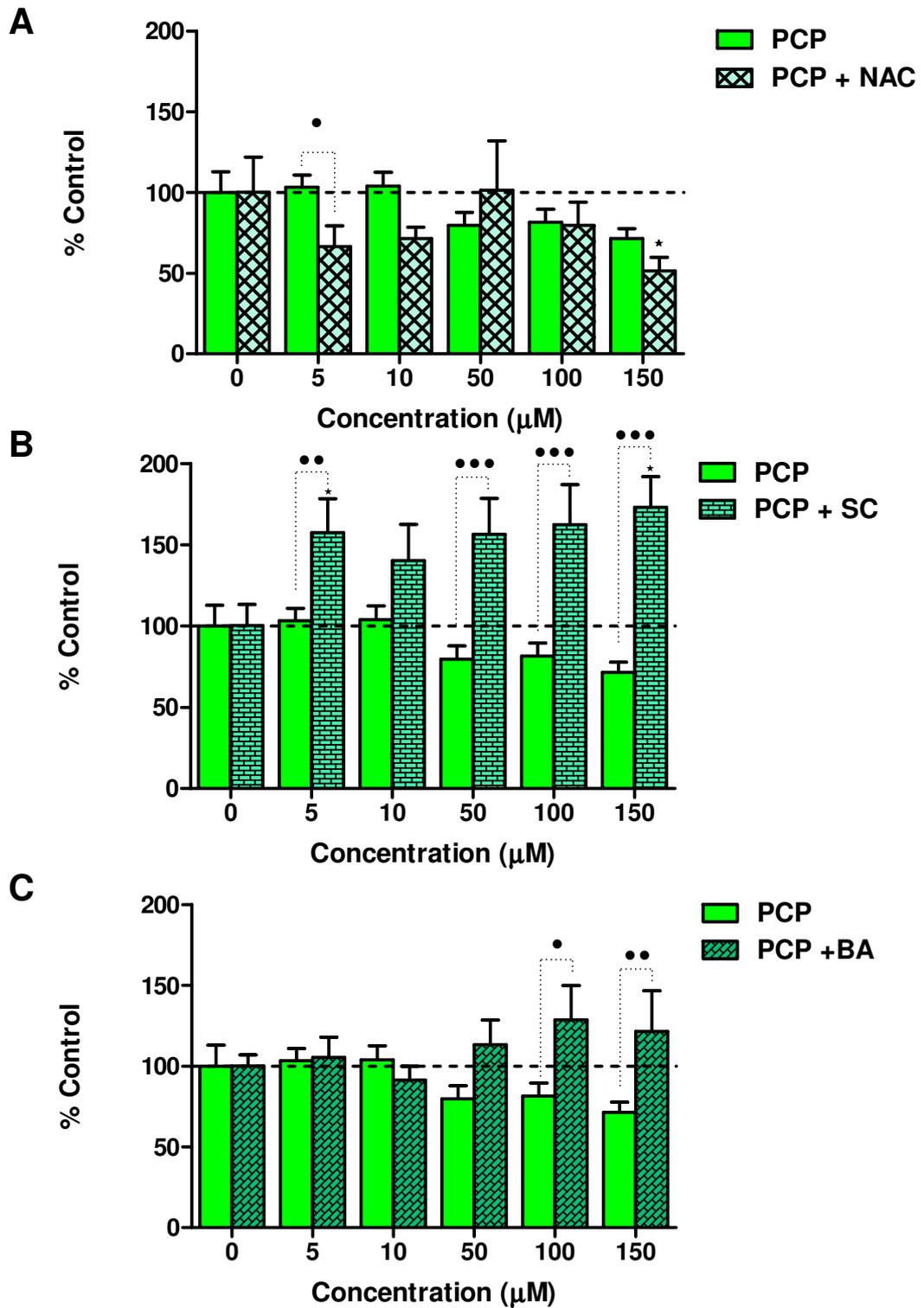


Figure 36. Necrosis determined by a decrease in membrane integrity in HepG2 cells directly exposed to PCP compared to necrosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control are indicated by *, p values < 0.05. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration is indicated by •, ••, ••• representing p values < 0.05, 0.01, 0.001, respectively.

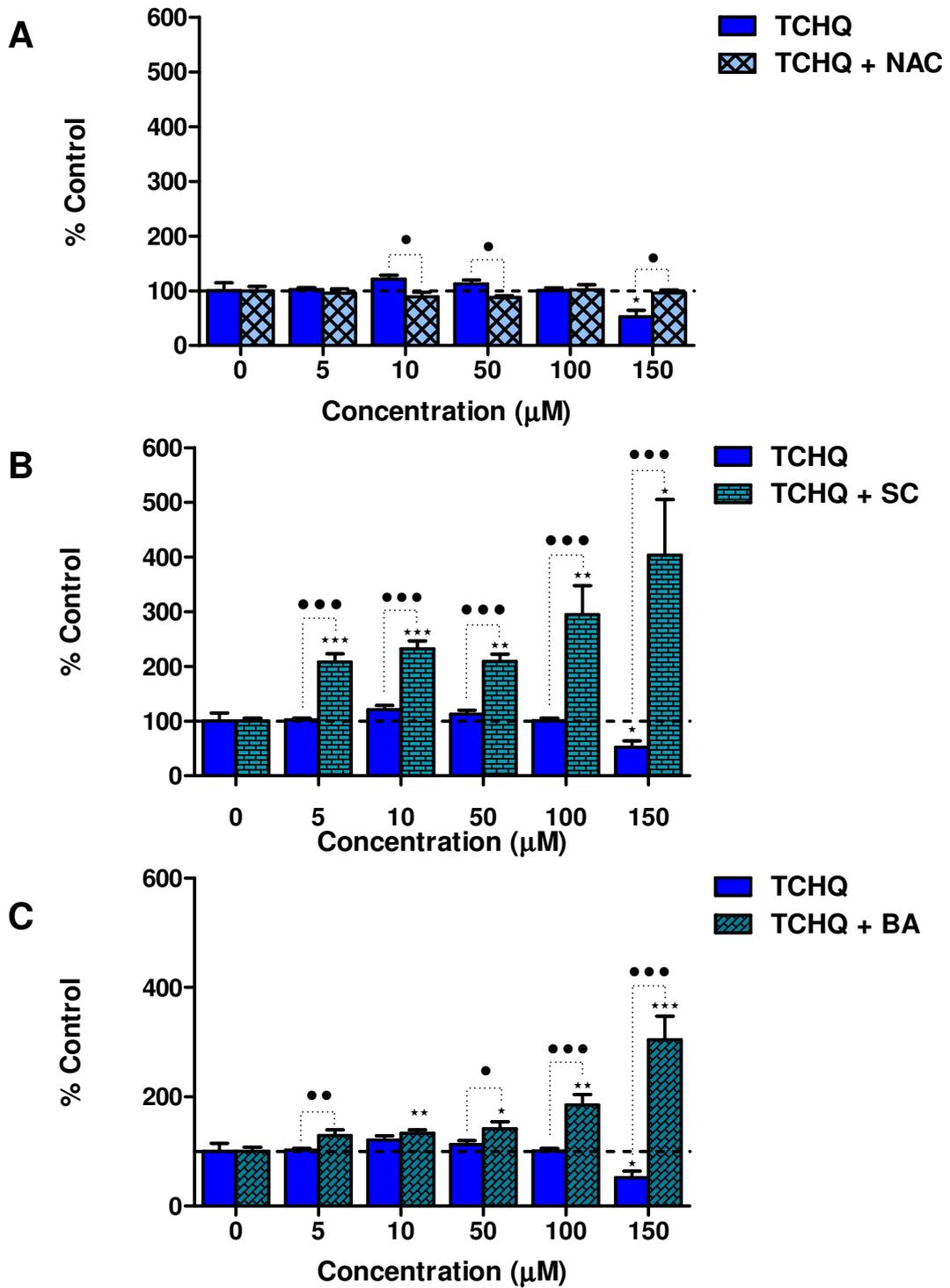


Figure 37. Necrosis determined by a decrease in membrane integrity in HepG2 cells directly exposed to TCHQ compared to necrosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCHQ exposure. Significant differences from the vehicle control are indicated by *, ** and *** representing p values < 0.05, 0.01 and 0.001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by •, •• and ••• representing p values < 0.05, 0.01 and 0.001, respectively.

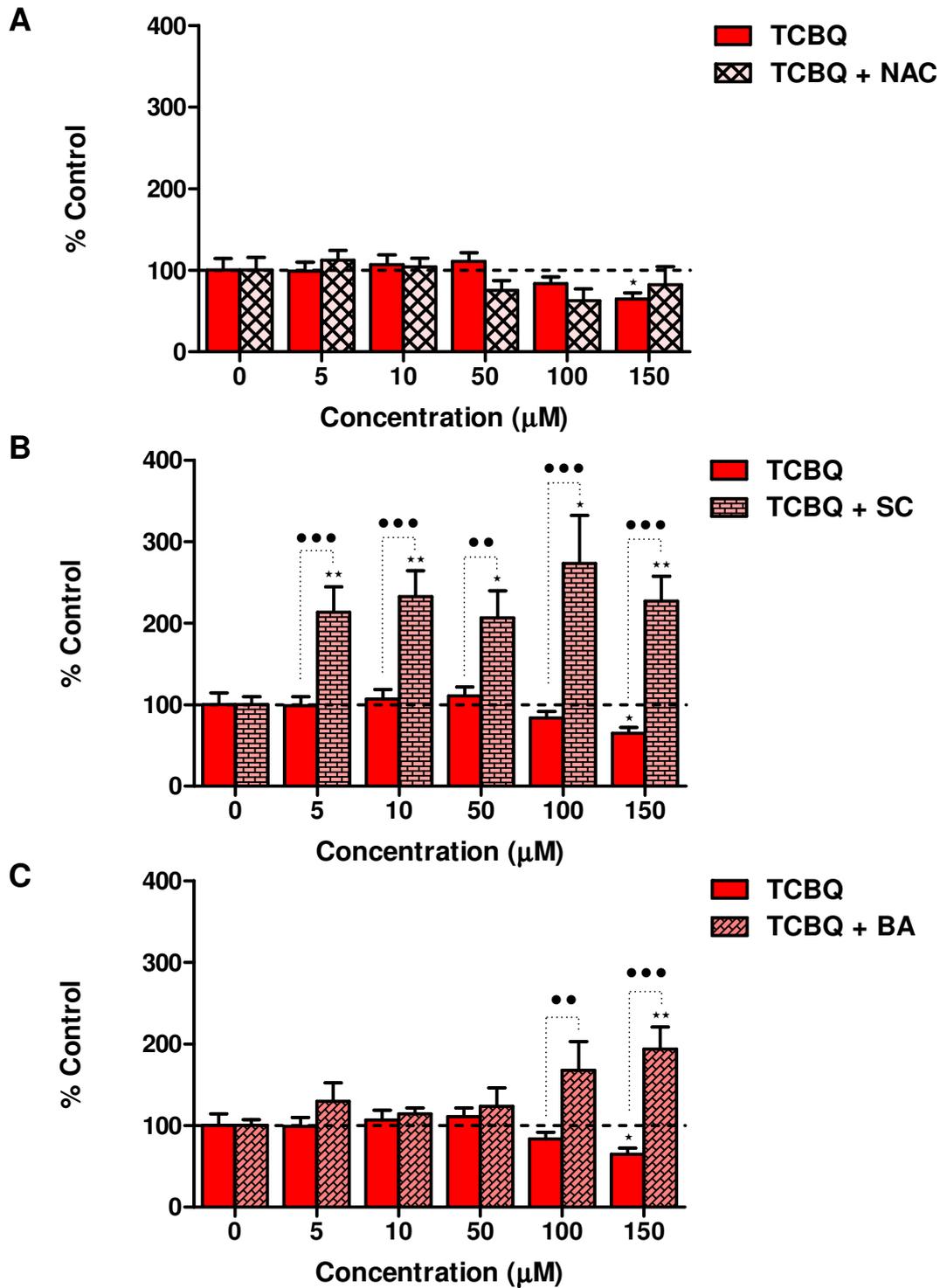


Figure 38. Necrosis determined by a decrease in membrane integrity in HepG2 cells directly exposed to TCBQ compared to necrosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control is indicated by * and ** representing p values < 0.05, 0.01, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by •• and ••• representing p values < 0.005 and 0.0001, respectively.

3.2.6 Apoptosis

Pre-treatment of cells with NAC caused a significant ($p < 0.05$) increase in caspase-3 activity when exposed to all test concentrations of PCP compared to cells exposed to PCP alone (Figure 39A). Pre-treatment with SC and BA did not alter caspase-3 activity in cells exposed to all test concentrations of PCP (Figure 39B and C).

Pre-treatment of cells with NAC had little effect on caspase-3 activity when exposed to all test concentrations of TCHQ compared to cells only exposed to TCHQ without pre-treatment (Figure 40A). Pre-treatment of cells with both SC and BA caused slight increases in caspase-3 activity when exposed to all test concentrations of TCHQ (Figure 40B and C). These increases were, however, only significant ($p < 0.05$) when cells were exposed to 100 μM TCHQ for both SC and BA pre-treated cells.

Pre-treatment of cells with NAC, as well as SC and BA, showed little change in caspase-3 activity when exposed to all test concentrations of TCBQ (Figure 41). Caspase-3 activity in cells pre-treated with NAC significantly ($p < 0.01$) decreased from the vehicle control when exposed to 100 and 150 μM of TCBQ.

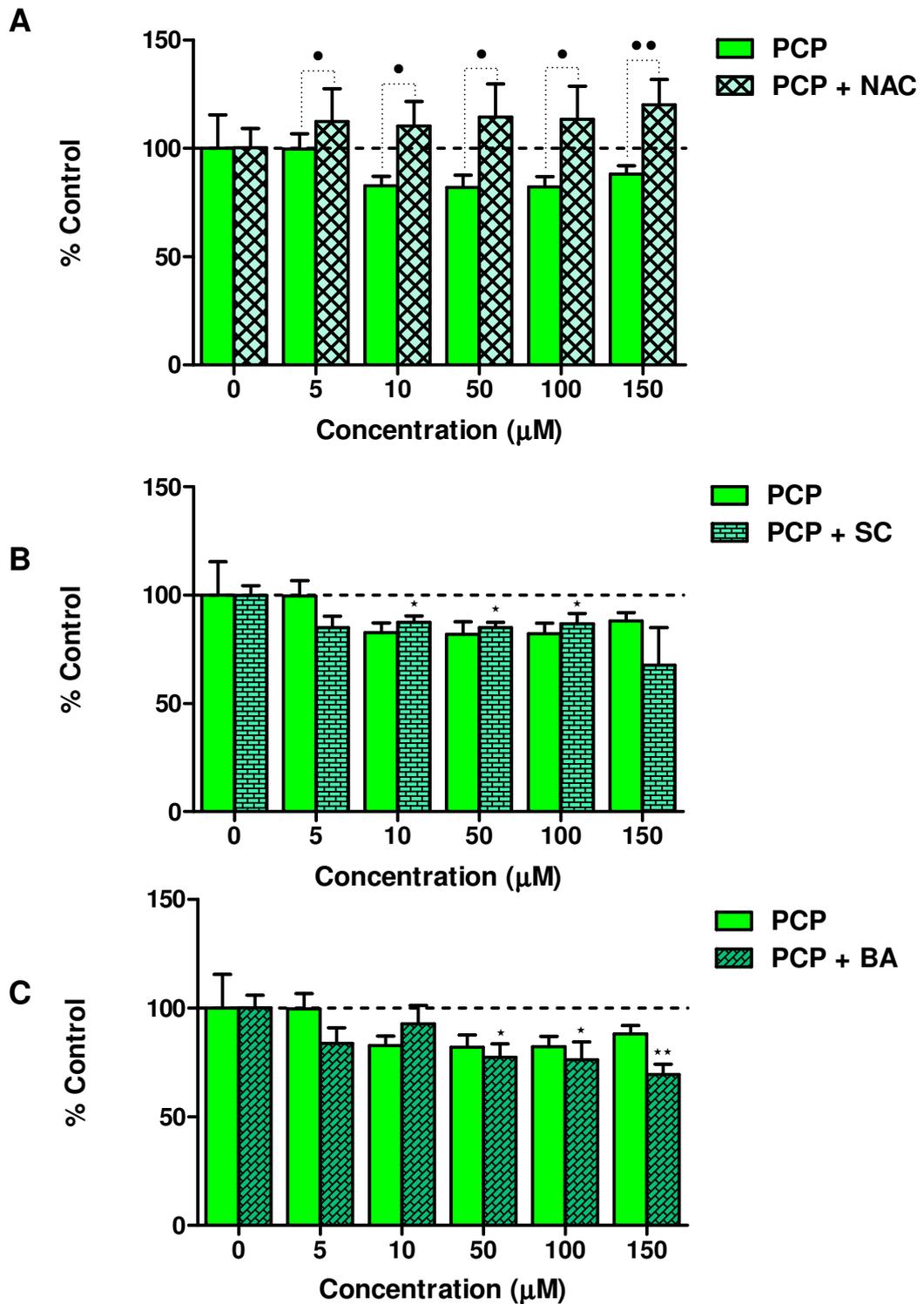


Figure 39. Apoptosis determined by an increase in caspase-3 activity in HepG2 cells directly exposed to PCP compared to apoptosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to PCP exposure. Significant differences from the vehicle control are indicated by * and ** representing p values < 0.05 and 0.01, respectively. Significant difference between groups exposed directly to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by • and •• representing p values < 0.05 and 0.01, respectively.

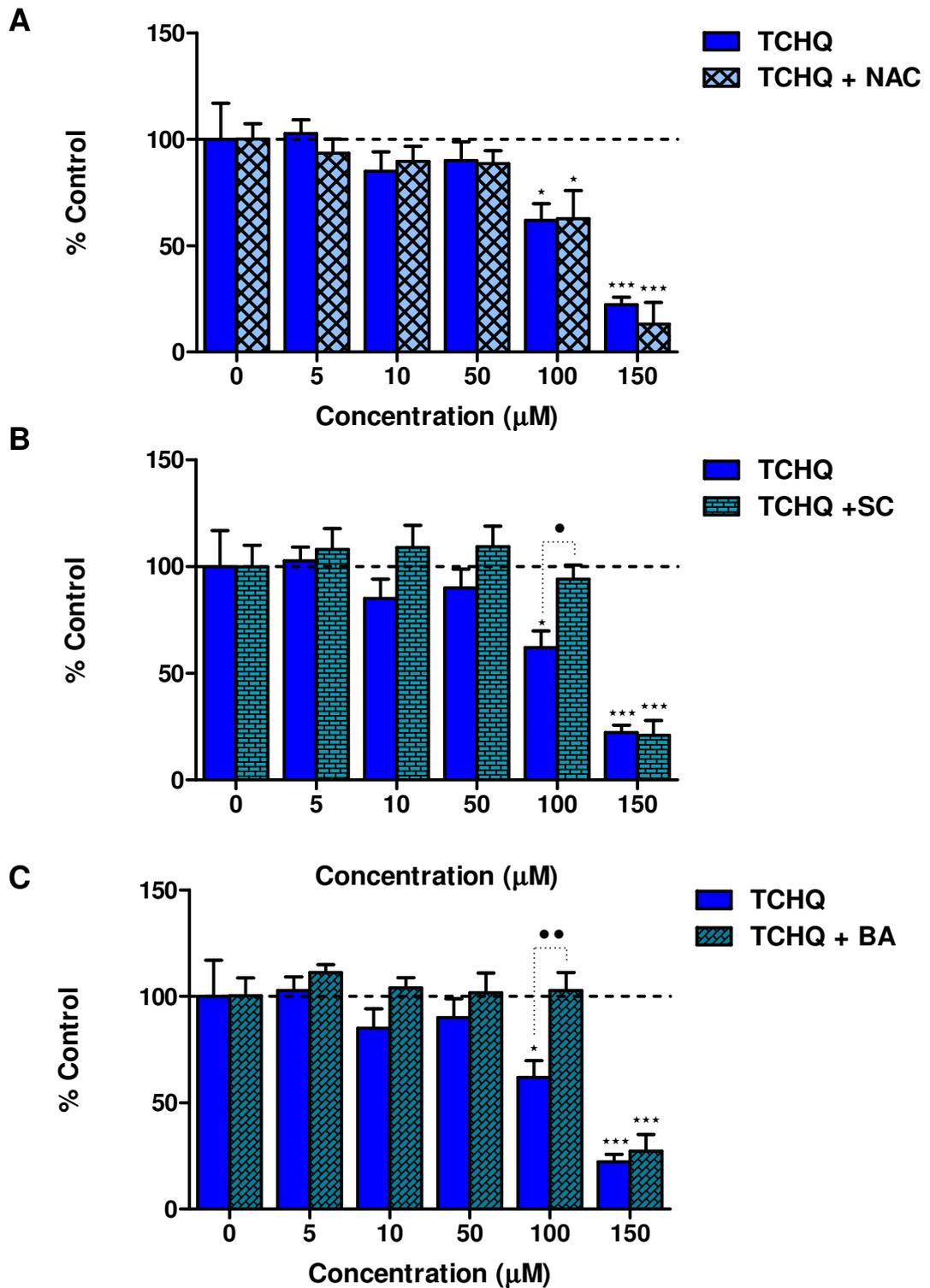


Figure 40. Apoptosis determined by an increase in caspase-3 activity in HepG2 cells directly exposed to TCHQ compared to apoptosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCHQ exposure. Significant differences from the vehicle control are indicated by * and *** representing p values < 0.05 and 0.001, respectively. Significant differences between groups directly exposed to pesticides and groups first pre-treated with NAC or plant extracts for each concentration are indicated by • and •• representing p values < 0.05 and 0.01, respectively.

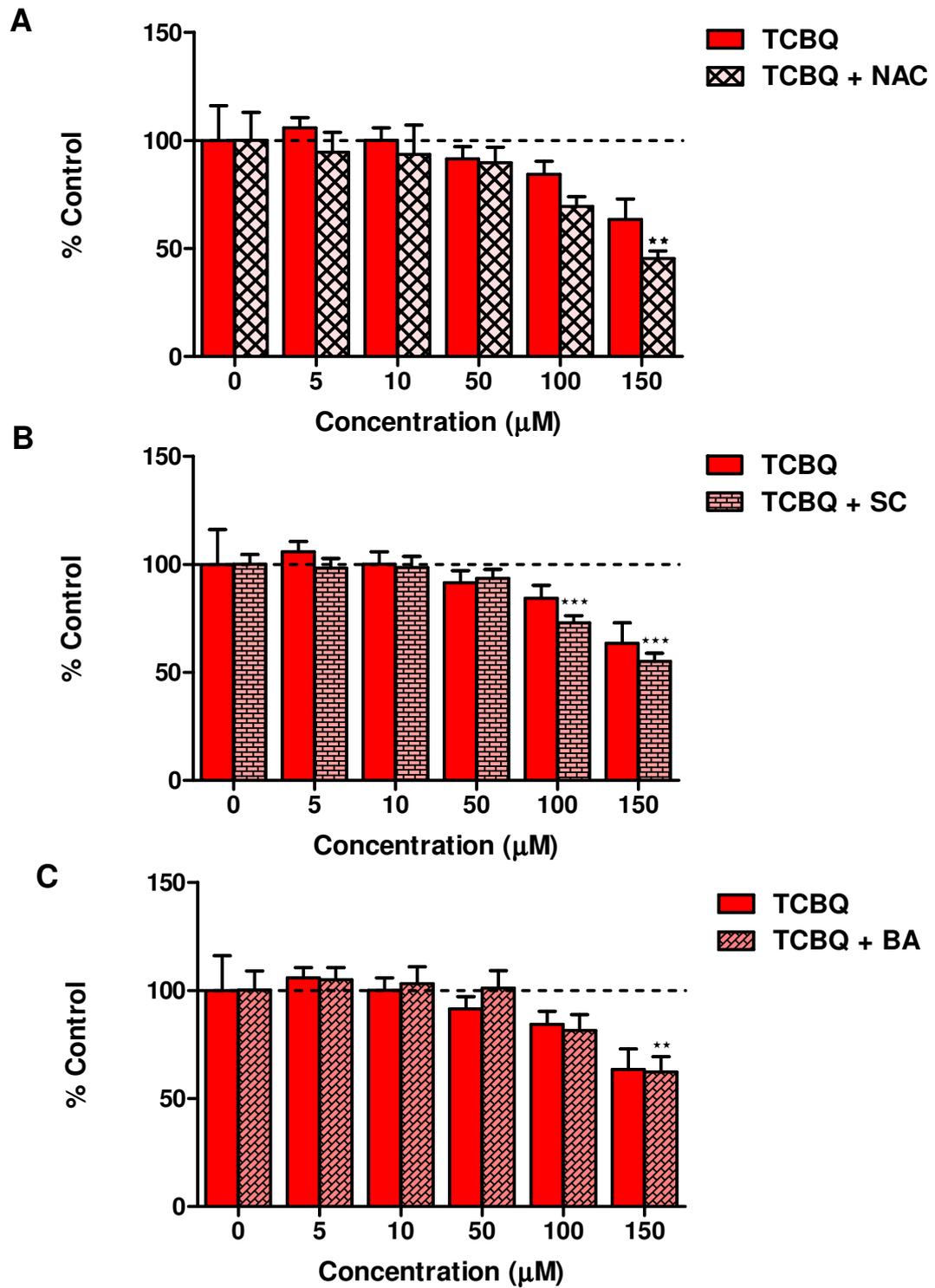


Figure 41. Apoptosis determined by an increase in caspase-3 activity in HepG2 cells directly exposed to TCBQ compared to apoptosis in HepG2 cells pre-treated with (A) NAC (B) SC or (C) BA prior to TCBQ exposure. Significant differences from the vehicle control are indicated by ** and *** representing p values < 0.01 and 0.001 respectively.

Chapter 4 - Discussion

4.1 Cell viability

Many cell viability assays exist, each with their own advantages and disadvantages. Of these assays the MTT assay and the NRU (3-amino-m-dimethylamino-2-methylphenazine hydrochloride) assay are commonly used. Both are easy to perform and the reagents required are usually readily available in most cell culture laboratories.

PCP is a well-known uncoupler of oxidative phosphorylation¹³³ and therefore directly affects the mitochondria. As the MTT assay is dependent on mitochondrial function, this could result in over estimation of the growth inhibitory effect of the pesticide. Furthermore, it has been shown that certain herbal extracts are capable of reducing MTT in the absence of living cells, ultimately resulting in false positive results¹³⁴. The NRU assay has also been proven to be more sensitive in revealing PCP toxicity than the MTT assay^{133,135}. For these reasons, the NRU assay is more suitable than the MTT assay, for use in the present study.

PCP has proven to have toxic effects on a number of cell lines. These include *Carassius carassius* primary hepatocytes¹³, Vero (monkey kidney fibroblasts)¹¹⁰, CGN (rat cerebellar granule neurons)¹³⁶, 3T3 (mouse embryonic fibroblast)¹¹⁰, rat Sertoli cells¹³⁷, AML-12 (mouse hepatocytes)⁴², Jurkat T-cells²⁶, HeLa (human ardenocarcinoma), HepG2 (human hepatoma)^{39,41,46}, Chang (human liver cells)³⁷, T-24 (human bladder cells)³⁷ as well as human lymphocytes and fibroblasts⁴⁸.

PCP showed significant cytotoxicity in AML-12 mouse hepatocytes⁴² and *Carassius carassius* hepatocytes¹³. Dorsey *et al.*⁴² reported a cell viability of $100 \pm 0.0\%$, $173.0 \pm 0.4\%$, $116.0 \pm 0.4\%$, $87.0 \pm 0.2\%$, $60.0 \pm 0.2\%$, and $48.0 \pm 0.1\%$ in AML-12 mouse hepatocytes exposed 0, 1.93, 3.87, 7.75, 15.5 and 31.0 $\mu\text{g/mL}$ (0, 7.25, 14.53, 29.09, 58.20, 116.4 μM) PCP, respectively. Dong *et al.*¹³ reported *Carrassius carrasius* hepatocyte's viability to be $78.0 \pm 9.6\%$, $54.7 \pm 4.1\%$ and $38.3 \pm 5.4\%$ when exposed to 1, 10 and 100 μM PCP, respectively. Chang liver cells were more resistant to the toxic

effects of PCP, only showing decrease in cell viability when exposed to concentrations of PCP $> 500 \mu\text{M}$ ³⁷. It is thus evident that not only does PCP have varying effects on different cell lines, but that length of exposure and concentration also play a major role in the extent of cytotoxicity.

In the present study both PCP and its metabolites were found to be acutely cytotoxic to HepG2 cells after a 24 h exposure period. PCP-induced cytotoxicity demonstrated a classical dose-response with cell viability gradually decreasing when exposed to increasing concentrations of PCP. TCHQ and TCBQ showed similar toxic profiles at concentrations $\geq 50 \mu\text{M}$ where a dose-response effect was evident.

In the present study, IC_{50} values indicated that PCP ($\text{IC}_{50} = 68.05 \mu\text{M}$) was most toxic followed by TCBQ ($\text{IC}_{50} = 129.40 \mu\text{M}$) and then TCHQ ($\text{IC}_{50} = 144.00 \mu\text{M}$). Cytotoxicity studies performed in HepG2 cells exposed to PCP and TCHQ are presented in Table 6. The IC_{50} for 24 h exposure to PCP determined by Jiang et al.⁴¹ is comparable with the IC_{50} determined in the present study. Wang et al.⁴⁶ found the concentration of PCP required to cause 50% cell death to be 5 times higher than that required for TCHQ in HepG2 cell. The reason for the differences in the present study to other reported results could be attributed to the method used. The trypan blue exclusion method is reported to be inaccurate in the identification of dead cells and thus underestimates cell death^{139,140}. Time is also critical since viable cells will begin to take up trypan blue after a certain period^{139,140}.

The decrease in toxicity observed for PCP in the aforementioned studies, compared to the present study, can be attributed to the fact that the MTT assay was used. Even though PCP is an uncoupler of oxidative phosphorylation, it has been shown that the NRU assay is more sensitive to detect PCP toxicity than the MTT assay¹³³. It has been suggested that this sensitivity is due to alterations in physical properties of membranes caused by PCP, which leads to disturbance in lysosomal membrane integrity, pore formation and ion pumps thus resulting in disruption of lysosomal acidity^{133,135}.

NAC had negligible effects on overall cell viability in cells exposed to PCP, TCHQ as well as TCBQ. This is contradictory to a study performed by Wang et al.¹⁴¹ where incubation with 30 mM NAC for 2 h significantly decreased toxicity induced by TCHQ in 3T3 cells. The difference could be attributed to the higher concentration and longer exposure time of NAC used by Wang et al.¹⁴¹ compared to that of the present study. The different cell lines used would also explain the discrepancy in the results obtained. BA and SC significantly increased cell viability in all cells exposed to higher concentrations of PCP. This increase in cell viability may be attributed to the corresponding decrease in CYP1A1 activity in cells pre-treated with SC and BA prior to PCP exposure. The biggest increase in IC₅₀ value was observed in cells pre-treated with BA, the greatest decrease in CYP1A1 however occurred in cells pre-treated with SC. This suggests that CYP1A1 may not be the primary mechanism of PCP cytotoxicity. SC counteracted low-level toxicity of PCP and its metabolites up to concentrations of 10 µM demonstrated by viability not dropping below 100%. This suggests that early treatment of PCP poisoning is key in counteracting its toxicity. This is supported by the fact that the plant extracts did not increase viability of cells treated with the metabolites as extensively as in cells exposed to PCP.

Table 6. *In vitro* cytotoxicity studies performed in HepG2 cells exposed to PCP and TCHQ.

Compound	Concentration	Cell line	Exposure time	Cytotoxicity assay	Result	Reference
PCP	250µM	HepG2	24 h	Tryptan Blue exclusion method	Cell viability < 70 %	Wang et al. ³⁸
			48 h		Cell viability < 50 %	
TCHQ	100µM		24 h		Cell viability < 60 %	
			48 h		Cell viability < 44 %	
PCP	Serial doses 0 - 100µg/ml (0 – 375.46 µM)	HepG2	48 h	MTT assay	LC ₅₀ = 23.56.µg/ml (88.46 µM)	Dorsey et al. ³⁹
PCP		HepG2	24 h	MTT assay	IC ₅₀ =28.53 mg/L (107.12 µM)	Jiang et al. ⁴¹
			48 h		IC ₅₀ =17.59 mg/L (66.04 µM)	

4.2 CYP 1A1 activity

The Ethoxyresorufin-O-deethylase (EROD) assay has widely been used as a qualitative and somewhat quantitative measure of CYP1A1 activity. This assay works on the principle that CYP1A1 acts on the substrate 7-ethoxyresorufin and oxidizes the α -C-H to α -C-OH¹⁴². This product, in turn, spontaneously dissociates to resorufin, a fluorescent compound which can be quantified by fluorometric methods.

The first step in the induction of the CYP1A1 enzyme is the binding of a ligand to the Aryl Hydrocarbon Receptor (AHR) with subsequent activation and translocation from the cytosol to the nucleus^{143,144}. The AHR is known as a ligand-activated transcription factor and is a member of the basic helix-loop-helix (bHLH) family of DNA binding proteins³⁹. It is usually found in the cytosol as a heterotetrameric 9S complex with two heat shock protein molecules (2 × Hsp90) and other protein(s)^{70,145,146}. Hsp90 prevents transcriptional activation by AHR but keeps the receptor in a ligand binding conformation¹⁴⁶. After ligand binding, Hsp90 dissociates and AHR translocates to the nucleus, aided by the Aryl hydrocarbon nuclear translocator (ARNT), after which this heterodimer interacts with the 5'-GCGTG-3' DNA sequence of the Xenobiotic Responsible Element (XRE) within the promoter region of the CYP1A1 gene^{143,146}. This allows for the up-regulation of transcription and increase in CYP1A1 mRNA and finally enzyme levels¹⁴³.

Among the ligands that are able to bind the AHR are polycyclic aromatic hydrocarbons, which include both PCP and its metabolites. Dorsey *et al.*³⁹ reported that CYP1A1 activity increased in HepG2 cells exposed to increasing concentrations of PCP up to 50 μ g/ml (approximately 188 μ M PCP). Zhang *et al.*²⁰ and Han *et al.*⁴³ both noted that PCP increased EROD activity in hepatic microsomes of *Carassius carassius* with increasing concentrations as well as increasing exposure time. Zhang *et al.*²⁰ reported that the percentage induction of EROD activity was 121% and 125% as well as 152% and 180% that observed in the water control, in groups exposed to 20.4 μ g/L (approximately 0.075 μ M) and 40.7 μ g/L (approximately 0.15 μ M) PCP for 7 and 15 d treatment, respectively. Han *et al.*⁴³ reported an induction of 122%, 183%, and 195% as well as 176%, 243%, and 261% when cells were exposed to 2.0, 4.0, and 6.0 mg/L (approximately 7.5, 15.0

and 22.5 μM , respectively) PCP for 24 and 72 h, respectively. The results of Zhang *et al.*²⁰ cannot be directly compared to the results in the present study, as a longer exposure time, a lower concentration of PCP and different cells were used. However, both studies demonstrate that PCP induces CYP1A1 activity. Concentrations of PCP tested by Han *et al.*⁴³ were also much lower than the concentrations in the present study, however, both studies reported an increase in EROD activity after exposure to PCP. The CYP1A1 activity seen in the present study is also supported by the results of Dorsey *et al.*³⁹ who reported an increase in XRE activity in cells exposed to PCP. Dorsey *et al.*³⁹ therefore concluded that the increase in CYP1A1 activity was due to the affinity of the AHR for PCP and subsequent binding to the XRE³⁹. The induction of CYP1A1 activity observed in HepG2 cells exposed to PCP, as well as its metabolites, in the present study can thus be attributed to the binding of the pesticides to the AHR subsequently inducing CYP1A1 activity as depicted in Figure 42. As CYP1A1 is one of the major enzymes involved in phase I metabolism it can be concluded that the phase I biotransformation reaction is active in HepG2 cells exposed to not only PCP but also, to a lesser extent, TCHQ and TCBQ. This finding is supported by Dorsey *et al.*³⁹.

Although both TCBQ and TCHQ induced CYP1A1 in HepG2 cells, the induction was far less extensive than the induction observed in cells exposed to PCP. It has been shown that oxidative stress caused by ROS generation, especially H_2O_2 , down regulates CYP1A1 gene expression¹⁴⁴. As was shown in the results, both metabolites caused generation of high levels of ROS, which may have down regulated CYP1A1 gene expression thus explaining the decrease in CYP1A1 activity compared to that observed in PCP treated cells. To our knowledge, this is the first report of the CYP1A1 inducing effects of both TCHQ and TCBQ in HepG2 cells.

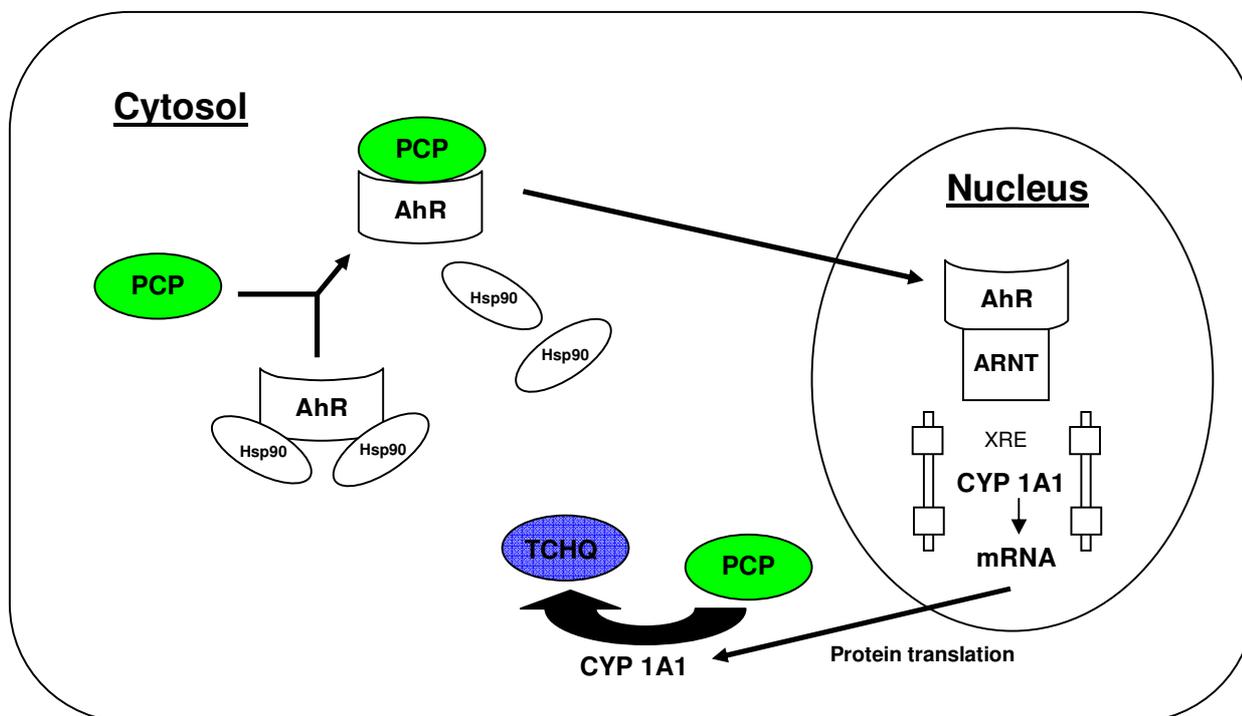


Figure 42. Proposed mechanism of PCP induced CYP1A1 activity.

The CYP1A1 induction observed in HepG2 cells exposed to PCP did not follow a dose-response pattern as reported by Dorsey *et al.*³⁹. In cells exposed to 5, 10 and 50 μM PCP, CYP1A1 induction was observed to be at its maximum. This can be attributed to the saturation of the AHR binding sites. It has been reported that the maximum observable response varies depending on the inducer¹⁴⁷. In cells exposed to 100 and 150 μM PCP the CYP1A1 activity was far less extensive than in cells treated with lower concentrations of the compound. It has been shown that CYP1A1 activity increases in a dose-dependent manner only to a certain extent, after which it decreases¹⁴⁷. This decline in CYP1A1 has been attributed to metabolism of resorufin by DT-diaphorase, inhibition of heme biosynthesis or reduced activity of NADPH-Cytochrome p450 reductase¹⁴⁸. In the present study, however, the decrease in CYP1A1 activity in cells exposed to 100 and 150 μM of PCP compared to cells exposed to lower levels of PCP can be attributed to the loss in cell viability observed at the higher concentrations. A

similar effect was also reported by Dorsey *et al.*³⁹ where a decrease in CYP 1A1 activity was observed in cells treated with 100µg/ml PCP (approximately 375.5 µM) compared to cells treated with 50µg/ml PCP (approximately 187.7µM). This may also be attributed to a decrease in viability at the higher concentration. Cells exposed to TCHQ and TCBQ showed a dose-response pattern in CYP1A1 induction. As the CYP1A1 induction was far less than observed in cells exposed to PCP, it is possible that the AHR receptor was not saturated making a dose-response pattern possible. A decrease in CYP1A1 activity was also seen in cells exposed to concentrations of > 50 and > 100 µM TCHQ and TCBQ, respectively. This again can be attributed to the loss in cell viability also seen at these concentrations.

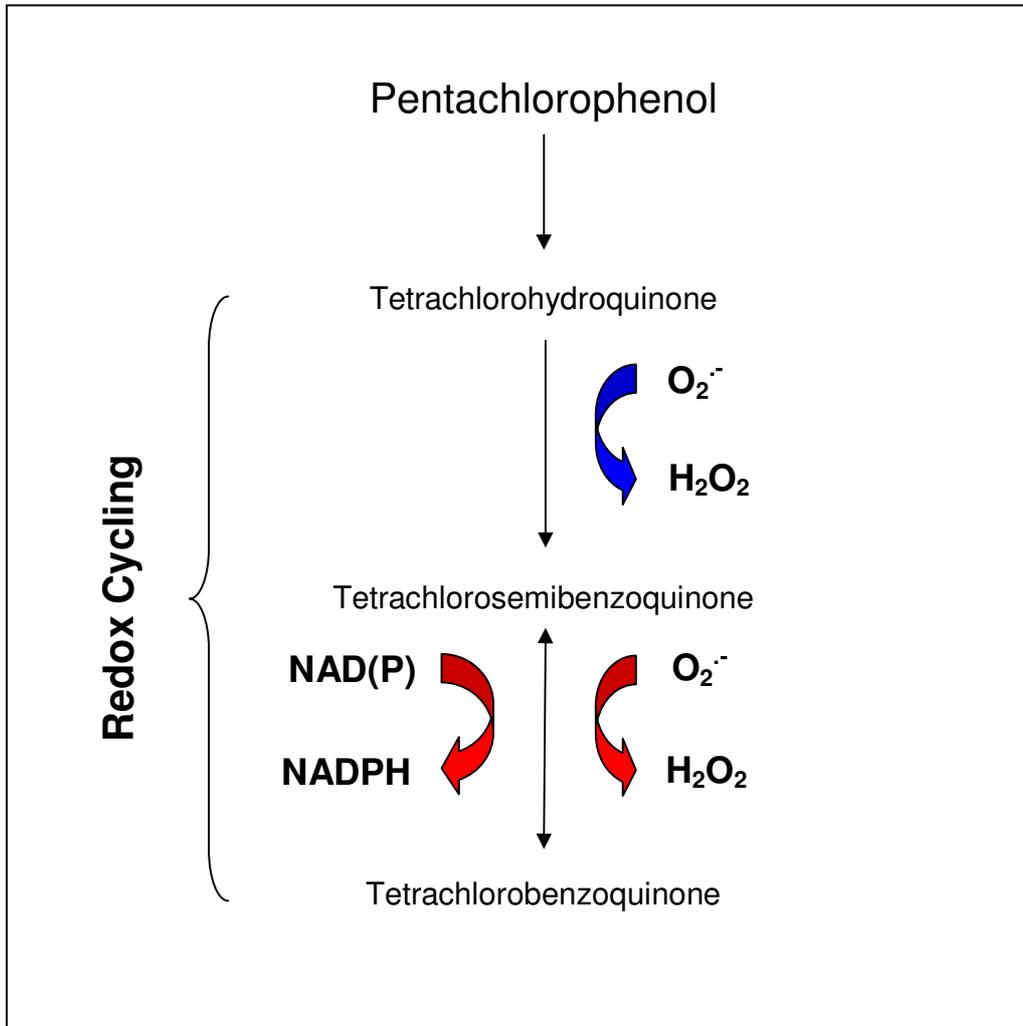
Pre-treatment of cells with NAC as well as BA and SC caused a significant decrease in PCP-induced CYP1A1 activity. However, in cells exposed to TCHQ and TCBQ a general pattern could be seen where NAC, as well as the plant extracts, in combination with the test compounds, induced CYP1A1 activity. However, in cells exposed to concentrations of TCHQ and TCBQ that greatly induced CYP1A1, NAC and the plant extracts still seemed to have a decreasing effect on the enzyme activity. Interestingly, NAC and both plant extracts caused both increases and decreases in CYP1A1 activity depending on the concentration of TCBQ and TCHQ. This phenomenon can be explained by the fact that the pre-treatments are partial agonists, competing with the pesticide for the AHR receptor, which upon binding does not activate the receptor to its full extent thus causing the observed CYP1A1 induction/inhibition depending on the concentration of the test compounds. From the results observed in the present study BA appears to be a more potent agonist than NAC and SC, as it showed greater CYP1A1 induction. It is also possible that the pre-treatment also affected the stability of the AHR-HSP90 complexes causing increased dissociation of AHR and increased transcription of CYP1A1. No other studies could be found where the effects of BA, SC or NAC on CYP1A1 activity in cells exposed to PCP and its metabolites were investigated.

4.3 Reactive oxygen species generation

ROS are partially reduced and highly reactive O₂ metabolites¹⁴⁹. Among these are short-lived entities such as hydroxyl (·OH), alkoxy (RO·) or peroxy (ROO·) radicals,

those which live slightly longer such as superoxide(O_2^-) and non radicals such as organic hydroperoxides (ROOH), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2)⁷². ROS are capable of interacting with cellular macro molecules and cause structural damage to proteins, lipids and DNA^{72,150}. They may cause changes in membrane integrity / permeability, activation of proteases and nucleases, lipid peroxidation, mitochondrial homeostasis, and altered gene expression⁸⁹. ROS generation thus plays an important role in a number of pathological conditions. Even though ROS are infamous for their toxic attributes, they have also been said to be vital participants in cell signalling and regulation^{72,149}. The difference between their beneficial and detrimental role is determined by the concentrations of ROS present. Under normal physiological conditions excess endogenous ROS, generated not only in the mitochondria via oxidative phosphorylation but also in other organelles, are scavenged by endogenous anti-oxidants such as GSH^{89,149}. However, when the concentration of ROS increases extensively, due to exogenous ROS species, the natural balance in the oxidant-antioxidant system is disturbed, resulting in a state of oxidative stress. The hepatotoxicity of a large number of xenobiotics can be attributed to the large amounts of ROS generated during their metabolism.

Many authors have stated that the metabolism of PCP generates ROS^{19,27,38}. Auto-oxidation and/or enzyme-mediated oxidation of the quinol metabolites of PCP, such as TCHQ, to its respective semi-quinones and quinones, namely TCBQ followed by reduction of these quinones cause a redox cycling cascade which generates ROS mainly in the form H_2O_2 ^{27,38}. The metabolism of PCP is depicted in Figure 40. This explanation correlates with the results in the present study where no ROS generation was seen in cells exposed to PCP, as depicted in Figure 43, whilst extensive ROS generation was seen in cells exposed to TCHQ and especially TCBQ. Dong *et al.*¹³ also



(Adapted from Lin et al.¹⁷)

Figure 43. Redox cycling during the metabolism of PCP to TCHQ and TCBQ.

reported an increase in ROS generation in primary cultures of *Carassius carassius* hepatocytes when exposed to 1, 10 and 100 μM PCP for 8h. The deviations concerning ROS generation compared to the present study may be attributed to the fact that different cell lines were employed.

Wang *et al.*³⁸ reported a time dependant increase in intracellular ROS levels in NIH3T3 (a mouse embryonic fibroblast cell line) cells treated with 25 μM TCHQ. Using the salicylate hydroxylation assay TCHQ and TCBQ have been reported to produce $\bullet\text{OH}$ in two separate studies conducted by Zhu *et al.*^{151,152}. It has also been said that ROS mediated DNA damage is the predominant type of DNA damage induced by TCHQ and TCBQ²⁷ and that oxygen species may be involved in the mechanism of TCHQ toxicity³⁸. It is evident that the results of the aforementioned studies conducted on TCHQ and TCBQ correlate with the findings of the present study.

A decrease in ROS, although not significant, was evident in cells exposed to 10, 50 and 100 μM PCP. It could be hypothesized that slight ROS generation may have occurred in these cells activating the endogenous anti-oxidant defence system resulting in the decrease in ROS. In cells exposed to 150 μM PCP a slight increase in ROS was observed. It could be hypothesized that in these cells the increased ROS overpowered the anti-oxidant defence system.

A dose-response pattern in ROS was observed in cells exposed to TCHQ. ROS generation increased when treated with increasing concentrations of TCHQ up to 100 μM , after which it decreased. Although this decrease in ROS may be ascribed to a loss in cell viability, it is not very plausible as the incubation time for this assay was merely 3 h. Further investigation is required to explain this finding.

The extensive ROS generation caused by TCHQ and TCBQ is probably the main mechanism of their toxicity. Wang *et al.*¹⁴¹ reported that TCHQ causes GSH depletion, which may explain how the ROS generation observed in the present study can lead to cell death. The ROS generated by TCHQ and TCBQ may also have had detrimental

effects on mitochondria. In the present study however MMP was measured before ROS generation, it is therefore not known whether ROS were present in the cells at the time MMP was measured. It is thus also possible that the ROS generation originated in the mitochondria as a consequence of direct mitochondrial insult caused by the metabolites. ROS generation caused by TCHQ and TCBQ has been reported to cause DNA damage and adduct formation resulting in tumour formation and cancer^{17,27}. It has thus been stated that ROS generation during metabolism of PCP plays a key role in its carcinogenicity^{19,27}. Pre-treatment with SC and BA caused decreases in ROS in cells exposed to PCP with SC being the more potent radical scavenger.

It can be concluded that both plant extracts possess anti-oxidant properties. From the decrease in ROS seen in cells pre-treated with NAC prior to TCHQ and TCBQ exposure, it can also be said that NAC also possesses anti-oxidant properties. It has been reported that NAC protects cells against TCHQ toxicity due to it raising intracellular GSH¹⁴¹. Intracellular GSH in turn scavenges ROS explaining the decrease in ROS generation in the present study in cells pre-treated with NAC prior to TCHQ and TCBQ exposure. Even though NAC proved to have an anti-oxidant effect, SC and BA appeared to have greater anti-oxidant potential, with SC being the most potent. The compounds in BA responsible for its anti-oxidant effect have been reported to include fisetinidol-(4 α →8)-catechin-3-gallate and bis-fisetinidol-(4 α →6 α ,4 α →8)-catechin-3-gallate, and to a lesser extent, monomeric flavo-3-ols (catechin, epicatechin and fisetinidol)⁹³. Anthocyanidins, gallic acid, ellagic acid are substances with known anti-oxidant potential¹⁵³⁻¹⁵⁶. These compounds are present in SC, therefore its anti-oxidant effect can be attributed to them.

4.4 Mitochondrial membrane potential

Mitochondria are vital components of mammalian cells and play critical roles in cellular physiology and homeostasis. Not only do they modulate energy production, they also play critical roles in reactive oxygen and nitrogen species homeostasis, calcium regulation, heme metabolism and apoptosis¹⁵⁷. They have also been reported to modulate signal transduction pathways and are involved in nuclear regulation¹⁵⁷. Mitochondrial dysfunction can thus seriously affect the normal functioning of an organism.

The mitochondrial lipophilic, cationic probe JC-1 is increasingly being used to determine MMP *in vitro* and has been shown to have higher sensitivity than common probes such as Rhodamine 123¹⁵⁸. Advantages of the method is that it allows for the identifications of populations with different mitochondria content, can be used to study mitochondria in a variety of conditions and that it has been validated by investigation MMP at the level of a single mitochondrion¹⁵⁸.

Mitochondria produce large amounts of ATP mainly via oxidative phosphorylation^{159,160}. Oxidative phosphorylation is a process which synthesizes ATP by using an electron transport or respiratory chain located in the inner mitochondrial membrane^{161,162}. This respiratory chain consists of three proton pumps namely complex I - NADH dehydrogenase, complex III - cytochrome c reductase and complex IV - cytochrome C oxidase. It also contains an enzyme complex, complex II - succinate dehydrogenase, which does not pump protons and a fourth reversible proton pump known as ATP synthase¹⁶¹. High energy electrons carried by NADH and FADH₂ are passed down the respiratory chain. The energy that is released when these electrons are passed from one complex to another is used to pump protons (H⁺) across the inner mitochondrial membrane from the mitochondrial matrix into the inter-membrane space¹⁶². The movement of protons into the inter-membrane space forms an electrochemical gradient. Backflow of H⁺ down this gradient, in turn, powers ATP synthase, which is responsible for catalyzing the conversion of ADP + Pi to ATP¹⁶². This process is called oxidative

phosphorylation as oxygen acts as the final acceptor of electrons and protons (H+) creating a molecule of water¹⁶².

The mitochondrial electrochemical gradient across the intermembrane space consists of a membrane potential and a very small pH gradient¹⁶³. This proton motor force is the central parameter which controls not only respiratory rate and ATP synthesis but also the generation of ROS¹⁶⁴. It, in turn, is controlled by electron transport and proton leaks¹⁶⁴. The MMP across the inner membrane has been linked to a variety of mitochondrial functions including: ATP synthesis, Ca²⁺ homeostasis, metabolite transport and the import of mitochondrial proteins¹⁶³. MMP has thus been used as an indicator of the health of the organelle in cells exposed to a variety of toxic compounds.

MMP changes in response to a variety of agents. Modest depolarization will occur when there is an increased ATP demand whilst slight hyperpolarisation will occur when there is a decreased ATP demand¹⁶³. Certain uncouplers of oxidative phosphorylation, however, are able to cause rapid and extensive changes in MMP. When oxidative phosphorylation is uncoupled there is an electron transfer and proton influx into the mitochondrial matrix independent of the phosphorylation of ADP¹⁵⁷. This can result from either damage to ATP-synthase or other complexes or via active or passive increased permeability of the inner-mitochondrial membrane¹⁵⁷. An increased influx of protons into the mitochondrial matrix results in a decreased MMP referred to as mitochondrial depolarization¹³³.

PCP is a well-known uncoupler of oxidative phosphorylation and its main mechanism of toxicity has been attributed to the resulting increase in aerobic metabolism and increasing heat production⁴⁰. It has been classified a protonophoric uncoupler, which depletes the proton gradient by translocating protons from the intermembrane space into the mitochondrial matrix¹⁶⁰. A study by Gravance *et al.*¹⁶⁵ reported a decrease in MMP in rat sperm mitochondria when exposed to increasing concentrations of PCP (0.1 and 1.0 µM PCP) for approximately 30 min. Han *et al.*⁴³ also showed significant dose-dependent decreases in MMP in liver microsomes of *Cyprinus carpio* (common carp) exposed to 2.0 mg/L (approximately 7.5 µM) PCP for 24 h and 2.0, 4.0 and 6.0 mg/L PCP for 72 h (approximately 7.5, 15.0 and 22.5 µM, respectively). Similarly, Dong *et*

*al.*¹³ showed a significant dose-dependent decrease in MMP in primary cultures of *Carassius carassius* (crucian carp) hepatocytes when exposed to 1, 10 and 100 μM PCP for 8h. Michalowics and Sicinska¹⁶⁶ reported an increase in the number of human lymphocytes characterized by a decreased MMP when exposed to 25 ppm (approximately 93.9 nM) PCP. Although in the above mentioned studies, different cells, different exposure times and different concentrations of PCP were used, these findings support the results of the present study in that PCP causes mitochondrial depolarization. Mitochondrial depolarization seen in cells exposed to PCP, in turn, supports the theory that PCP is an uncoupler of oxidative phosphorylation. In the present study significant decreases in MMP were seen in HepG2 cells exposed to 5 and 10 μM PCP. Higher concentrations of PCP did not show the same degree of depolarization. It has been shown that translocation of protons across the inner membrane space results in a compensatory increase in respiratory electron flow¹⁶⁰. Increased electron flow results in an increase of protons being pumped into the intermembrane space. This could result in a compensatory increase in MMP. This compensatory increase in mitochondrial potential may have occurred in the present study in the cells exposed to higher concentrations of PCP.

Significant mitochondrial depolarization also occurred in HepG2 cells exposed to 10-150 μM concentrations of both TCHQ and TCBQ. ROS is known to cause mitochondrial permeability transition (MPT), which leads to an influx of protons ultimately resulting in mitochondrial depolarization¹⁶⁷. The large amounts of ROS generated in cells exposed to TCBQ and TCHQ in the present study, therefore, may have resulted in mitochondrial depolarization. However MMP was measured before ROS generation and it is not known whether ROS was present during mitochondrial depolarisation. It can therefore not be eluded whether ROS caused the mitochondrial insult in the present study. TCBQ and TCHQ may have had direct effects on oxidative phosphorylation and / or mitochondrial membrane permeability resulting in mitochondrial depolarization. Pre-treatment of cells with BA and SC before TCHQ exposure did not have a significant effect on MMP compared to cells only exposed to TCHQ. A single study was found where the effect of TCHQ on MMP was investigated. Lin *et al.*¹⁶⁸ reported that when

NIH3T3 cells were treated with 10–100 mM TCHQ for 2 h, a decrease in fluorescence DiOC6 (3,3'-dihexyloxacarbocyanine iodide) intensity occurred with increasing concentrations of TCHQ. This finding correlates with the results of the present study.

As both SC and BA were shown to possess anti-oxidant activity in the present study, one would have expected to see an increase/recovery in membrane potential, due decreased ROS damage, in cells pre-treated with the plant extracts compared to cells exposed to TCBQ or TCHQ alone. However, as previously mentioned MMP was measured before ROS generation and therefore it is not known whether ROS caused mitochondrial insult. It should also be considered that the decreases in ROS after SC and BA pre-treatment could be attributed to the decrease in mitochondrial function. Flavanols found in BA such as catechin and epicatechin have been shown to induce apoptosis mainly through ROS generation and mitochondrial depolarisation in DU145 (human prostate cancer) cells¹⁶⁹. Anthocyanidins, components of SC, have been reported to decrease MMP in HepBs cells, human fibroblast cells, uterine carcinoma and colon adenocarcinoma cells^{170,171}. This suggests that these components in the plants may have caused further depolarization of the mitochondria in the cells exposed to TCBQ and TCHQ in the present.

Pre-treatment of cells with NAC prior to TCHQ or TCBQ exposure had little effect on MMP. The latter indicates that NAC pre-treatment provided no protection against mitochondrial insult caused by these pesticides.

4.5 Necrosis and apoptosis

Cell death constitutes an important event in cell biology¹⁷². Two main modes of cell death are distinguishable – apoptosis and necrosis^{172,173}. Apoptosis is said to be a physiological programmed cell death used in cell homeostasis^{172,173}. All cells in the human body possess an intrinsic capability to enter apoptosis and a resistance to this regulation may result in autoimmune diseases and/or cancer¹⁷². Apoptosis may also be triggered in stress conditions when cells are exposed to toxins or other physical or xenobiotic agents¹⁷³. Necrosis is said to occur due to acute cellular dysfunction caused by conditions of severe stress or toxic insult^{172,173}. Necrosis is a more passive process than apoptosis and is associated with rapid cellular ATP depletion¹⁷³. Although apoptosis may be considered as physiological or pathological in different circumstances, necrosis is always considered pathological¹⁶⁰. Even though apoptosis and necrosis are viewed as distinct opposing types of cellular death, they can be induced by the same toxins, the mechanism of cell death being determined by the concentration of the toxin¹⁷⁴. Furthermore, the mode of cell death can be manipulated by changes in ATP levels as well as caspase activation or inactivation by certain compounds¹⁷². In cells with high ATP levels, apoptosis would be favoured, however apoptosis can be transformed to necrosis by inactivation of caspases¹⁷².

The induction and execution of apoptosis is regulated by the co-operation of a variety of molecules including signal molecules, receptors, enzymes and gene regulating proteins^{173,175}. The caspase-cascade signalling system is among these molecules and plays a critical role in the process of apoptosis¹⁷⁵. Caspases are cysteine proteases which cleave after specific aspartate residues^{172,175}. Fourteen different caspases have been identified thus far and are divided into three subfamilies¹⁷⁵. These include subfamily I which are apoptosis activators: caspase-2, caspase-8, caspase-9 and caspase-10, subfamily II which are apoptosis executioners: caspase-3, caspase-6 and caspase-7 and subfamily III which are inflammatory mediators: caspase-1, caspase-4, caspase-5, caspase-11, caspase-12, caspase-13 and caspase-14.

There are two different apoptotic pathways; an extrinsic or death receptor-mediated pathway and an intrinsic or mitochondrion-mediated pathway¹⁷⁵⁻¹⁷⁷. The extrinsic

pathway is initiated when cell death signals are transmitted via the binding of an extracellular death ligand such as Fas Ligand (FasL) and tumour necrosis factor (TNF-2) to their corresponding death receptor Fas or TNF receptor (TNFR)¹⁷⁵⁻¹⁷⁸. In turn, these receptors form death inducing signalling complexes, which have adaptor proteins such as Fas-associated death domain (FADD) or TNFR- associated death domain (TRADD) as well as procaspase-8/-10^{175,178}. Together this forms a massive molecule complex known as the death-inducing signaling complex (DISC)^{175,179}. Formation of DISC leads to the activation of initiator caspases, namely caspase-8 and caspase-10, which, in turn, cleaves and activates executioner caspases, namely caspase-3, caspase-6 and caspase 7^{175,177-179}. Caspase-8 is also able to activate the intrinsic pathway by truncating a pro-apoptotic protein, Bid (a member of the Bcl-2 family), to its active form tBid which in turn triggers the mitochondrial or intrinsic pathway.

The intrinsic pathway is mediated by the mitochondria and is induced by chemicals, cellular stress or withdrawal of growth factors. In times of cellular stress pro-apoptotic proteins in the cytoplasm will be activated, which, in turn, induce the opening of the mitochondrial transition permeability pore with subsequent release of pro-apoptotic proteins such as cytochrome C, SMAC/DIABLO, AIF and EndoG from the mitochondria into the cytoplasm. Cytochrome C is the most well known of these^{175,179}. Upon binding with cytochrome C and in the presence of dATP or ATP oligomerisation of apoptotic protease activation factor-1 (Apaf-1) occurs¹⁷⁹. Oligomerized Apaf-1, together with dATP, procaspase-9 and cytochrome C results in the formation of a massive complex – the apoptosome¹⁷⁵. The apoptosome activates caspase-9 which in turn activates executioner caspases such as caspase-3^{175,177,179}. Activated caspase-3 is also able to activate pro-caspase 9 forming a positive feedback pathway¹⁷⁵. The mitochondrial mediated pathway is often controlled by pro-apoptotic and anti-apoptotic members of the Bcl-2 family¹⁷⁸. Pro-apoptotic members include Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, whilst antiapoptotic members include Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1¹⁷⁸.

In the present study the caspase-3 assay was used to detect apoptosis. Caspase-3 is one of the effector caspases and thus plays a direct role in the proteolytic digestion of

cellular proteins. Necrosis was determined by assessing membrane integrity using propidium iodide staining.

Since PCP did not significantly increase caspase-3 activity or decrease membrane integrity it cannot be concluded whether PCP induces apoptosis or necrosis. Two possible scenarios may have occurred. PCP could have either induced enough cell stress for apoptosis to occur or had a direct effect on the mitochondria, inducing release of pro-apoptotic proteins with the opening of the MPT pore allowing for the release of cytochrome C. As mentioned previously, upon release, cytochrome C forms the apoptosome with Apaf-1, dATP and procaspase-9. The apoptosome allows for activation of caspase-9 with subsequent activation of effector capsases such as caspase-3. In the present study the assay for the detection of caspase-3 may have been performed prematurely whilst the cells were still in the early stages of apoptosis prior to activation of caspase-3 and thus no increase in caspase-3 activity was observed. Alternatively, PCP may have caused a severe enough toxic insult which may have resulted in necrosis. It is possible that necrotic cells may have detached from the well surface and were subsequently washed off during the wash steps of the PI assay, and thus a decrease in necrosis could not be detected.

In cells exposed to both TCHQ and TCBQ the mode of cell death could also not be confirmed. Decreases in caspase-3 activity and PI fluorescence were seen in cells exposed to higher concentrations of TCHQ and TCBQ. These decreases were however not always significant and probably occurred as the result of a corresponding decrease in cell viability seen at these concentrations. As with PCP, TCHQ and TCBQ may have caused enough cell stress for apoptosis to occur. Their direct and indirect effects, due to their extensive ROS generating capabilities, on the mitochondria may also have induced release of pro-apoptotic proteins with the opening of the MPT pore allowing for the release of cytochrome C. Large amounts of ROS generation has been shown to cause inactivation of caspases¹⁷³. Due to the extensive ROS generated by TCHQ and especially TCBQ, caspase-9 and caspase-3 may have been inactivated possibly causing a shift to the necrotic pathway of cellular demise. Necrosis may not have been detected due to necrotic cells being detached from the well surface and washed off in

the wash step of the PI assay. Alternatively, PCP may have caused a severe enough toxic insult and / or ATP depletion (evident from the mitochondrial depolarisation), which may have resulted in necrosis.

Autophagy is another possible mode of cell death caused by PCP and its metabolites. However this is not likely in the case of PCP as it has been reported that it is an inhibitor of autophagy^{180,181}.

In certain instances small increases were seen in caspase-3 activity, these increases were significant at some concentrations compared to cells only exposed to the pesticides without pre-treatment. As no apoptosis was detected in cells exposed to the pesticides alone without pre-treatment with NAC or plant extract, no conclusions can be made as to whether NAC or plant extracts would have had a beneficial effect on apoptosis if it was in the process of occurring.

A minor, non significant decrease in PI fluorescence was observed in cells pre-treated with NAC and then exposed to 5 μ M PCP as well as 5 and 10 μ M TCHQ. These decreases only occurred when the PI fluorescence in the cells exposed directly to the corresponding pesticide concentration was higher than the vehicle control. The latter may indicate that NAC could possibly have had a beneficial effect, had necrosis been detected. A small increase in PI fluorescence was seen in cells pre-treated with NAC before exposure to 150 μ M TCHQ compared to cells exposed to 150 μ M TCHQ alone. There was no corresponding difference in viability at this concentration, thus the increase in necrosis could be attributed to experimental variability.

When cells were pre-treated with SC and BA a significant increase in necrosis was detected especially when exposed to higher concentrations of PCP and its metabolites. It can be postulated that since both SC and BA decreased ROS generation, that the apoptotic pathway would be favoured in these cells. However, even though the plant extracts somewhat decreased ROS generation they did not inhibit it completely and therefore sufficient H₂O₂ was still present in cells pre-treated with the plant extracts prior to pesticide exposure to inhibit caspases and favour necrosis. Secondly, it may be possible that the plant extracts directly induced necrosis. However, a corresponding

decrease in cell viability would have been expected in this case. There was, however, no significant decrease in cell viability in cells pre-treated with plant extracts prior to pesticide exposure making this theory questionable. Yet another possibility is that due to the exacerbated inhibition of MMP the mitochondria were unable to produce ATP. As apoptosis is an energy-dependant process, it could not occur in an ATP-reduced environment and thus necrosis was favoured.

The fact that necrosis could be detected in the above mentioned experiments supports the hypothesis mentioned earlier which suggests that the caspase-3 assay was performed pre-maturely.

Literature regarding apoptosis in animal cells is contradictory. Several studies reported PCP to induce apoptosis^{42,136,138,141,168}. Sai *et al.*¹⁸² reported PCP to inhibit apoptosis in v-myc transfected rat liver epithelial cells, whilst Yang *et al.*¹³⁷ reported that necrosis was the major mode of cell death evident in Rat sertoli cells. Wang *et al.*³⁷ found necrosis to be more evident in T-24 cells exposed to PCP whilst apoptosis was induced in T-24 cells exposed to TCHQ. Wang *et al.*³⁷ however found no evidence of apoptosis in Chang liver cells exposed to either PCP or TCHQ.

Literature suggests that concentration of PCP or TCHQ affects the mode of cell death. Wispriyono *et al.*²⁶ reported apoptosis in Jurkat cells when exposed to 20 μ M PCP or TCHQ, however, no apoptosis was evident at concentrations of PCP or TCHQ > 20 μ M. In contrast. Dong *et al.*¹³ reported that DNA fragmentation characteristic of apoptosis was absent in primary *Carassius carassius* hepatocytes exposed to 0.01 and 0.1 μ M PCP but present in cells exposed to 10 and 100 μ M PCP. It is evident that apoptosis and necrosis is sensitive to the cell line used. As no studies could be found on HepG2 cells no comparison regarding the effect of PCP or TCHQ on the mode of cell death in HepG2's could be deduced from literature.

4.6 Mechanism(s) of action of the test compounds

It is evident that the mechanism of toxicity of PCP differs from that of its metabolites, which have similar mechanisms of toxicity. In the present study PCP induced CYP1A1 activity and caused mitochondrial membrane depolarization with no ROS generation, whereas both TCHQ and TCBQ produced extensive ROS, less CYP1A1 but also mitochondrial membrane depolarization.

BA and SC pre-treatment caused definite inhibition of PCP-induced elevations in CYP1A1 activity at all test concentrations. Also, both the extracts caused considerable improvements in cell viability after PCP exposure. This suggests that CYP1A1 activity may be related to the mechanism of PCP toxicity. However, greatest inhibition of PCP-induced CYP1A1 activity resulted from SC pre-treatment and BA pre-treatment caused the largest increase in IC_{50} value after PCP exposure. For this reason, it can be argued that CYP1A1 induction is not the primary mechanism of PCP toxicity. PCP also caused mitochondrial depolarization. This may have led to the opening of the mitochondrial permeability transition pore and subsequent release of cytochrome C, activation of caspases and eventually apoptotic cell death. However, the exact mechanism of toxicity remains elusive. The proposed mechanism of hepatotoxicity for PCP is presented in Figure 44.

The fact that SC pre-treatment caused the highest increase in IC_{50} values for both TCBQ and TCHQ, coupled with the fact that SC was the most efficient radical scavenger of ROS, indicate that excessive ROS generation plays a central role in the mechanism of toxicity of both TCHQ and TCBQ. The excessive ROS generation may have caused mitochondrial insult that may have caused opening of mitochondrial permeability transition pore and release of cytochrome C which, in turn, may have activated apoptotic caspases. It is also possible that extensive ROS generation may have inactivated these caspases causing a shift to the necrotic mode of cell death. Depletion of ATP due to mitochondrial insult may also have led to necrosis rather than apoptosis. This, of course, is a hypothetical mechanism of action. The proposed mechanism of hepatotoxicity for TCHQ and TCBQ is presented in Figure 41.

4.7 Mechanism(s) of protection of the medicinal plants

BA and SC had definite beneficial effects on the viability of cells exposed to PCP, and both of its metabolites. One would expect that because both BA and SC are potent anti-oxidants and did inhibit ROS generation in the present study, their mechanism of protection may be attributed to their anti-oxidant activity. This is supported by improvements in the IC₅₀ values observed when cells were pre-treated with the plant extracts prior to exposure to TCHQ and TCBQ. NAC failed to produce similar hepatoprotective effects. Regarding TCHQ and TCBQ exposure, SC demonstrated greater radical scavenging capacity than BA. Also, SC proved to be more beneficial to the viability outcomes of cells following TCHQ and TCBQ exposure than BA. This suggests that the mechanism of hepatoprotection of SC is largely related to its radical scavenging capacity.

It can also be hypothesized that the increase in viability of cells exposed to PCP after pre-treatment with plant extracts may be attributed to the corresponding decrease in CYP1A1 activity. It is thus suggested that BA and SC may be useful in offering hepatoprotective effects against hepatotoxins which greatly induce CYPs and cause moderate ROS generation.

Although certain compounds in the plant extracts appeared to cause further mitochondrial depolarisation in cells exposed to PCP and TCHQ, there was not a further decrease in viability seen in these cells. It is therefore hypothesized that either the further decrease in MMP was not extensive enough to cause further decreases in cell viability or that the cells managed to recover from it and thus it had no detrimental effect on overall viability.

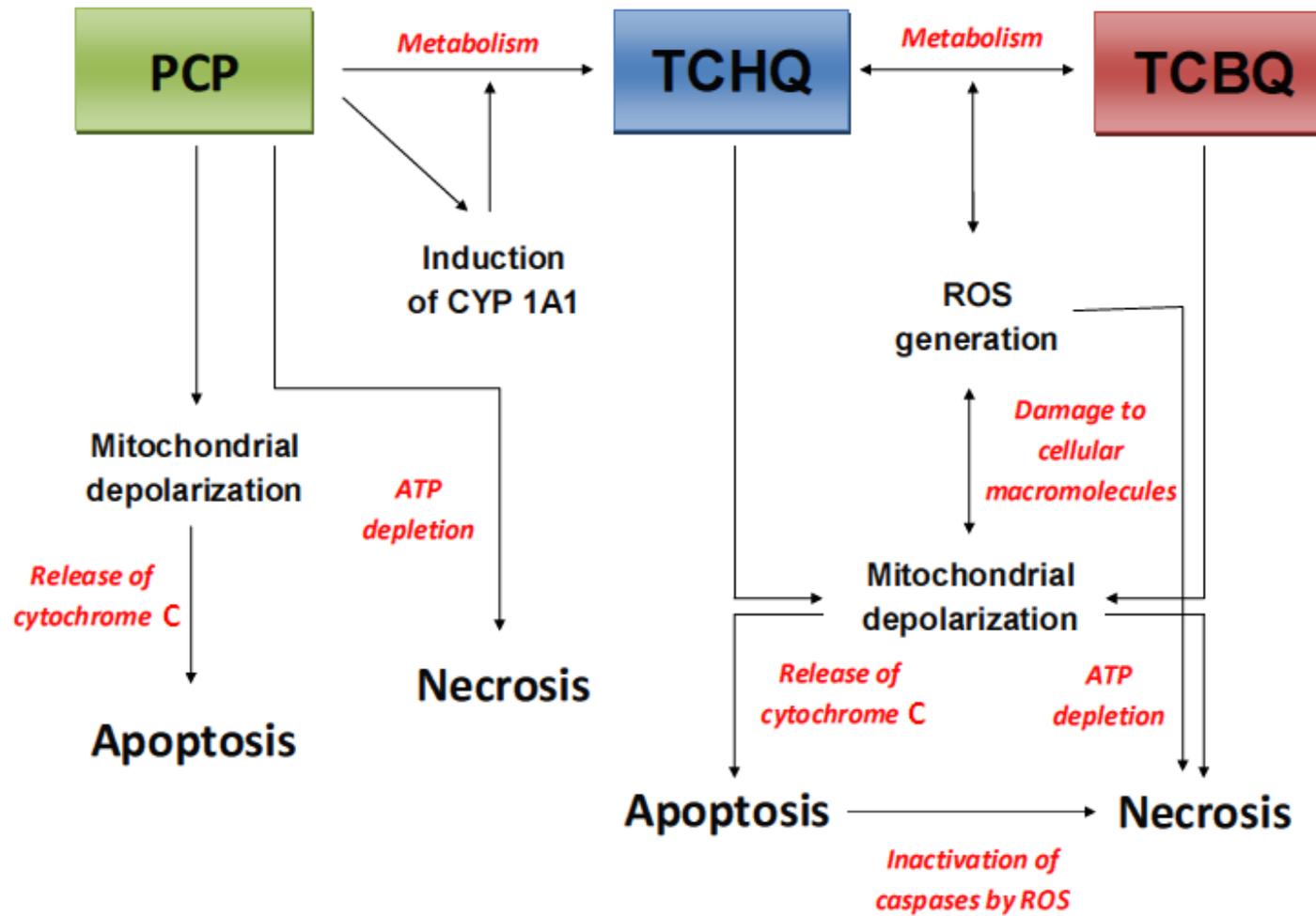


Figure 44. Proposed mechanism of toxicity of PCP and its metabolites.

Chapter 5 - Conclusion

The first aim of this study was to evaluate endpoints of hepatotoxicity induced by PCP and its metabolites in order to elucidate their possible mechanism of action. It was found that the mechanism of PCP toxicity differed to that of its metabolites. PCP was found to be more toxic than its metabolites. From the results in the present study it is proposed that PCP induces its own metabolism by inducing CYP1A1 activity. It also causes mitochondrial insult, which may lead to apoptotic cell death. With regard to TCHQ and TCBQ, it can be concluded that the extensive ROS generation produced caused damage to various macromolecules and could be the main cause of their toxicity. Both metabolites seem to have caused toxic insult to the mitochondria. This, in turn, may have resulted in the activation of apoptotic caspases. It is also possible that extensive ROS generation may have inactivated these caspases causing a shift to the necrotic mode of cell death. Depletion of ATP due to mitochondrial insult may also have led to necrosis rather than apoptosis.

A further aim was to investigate NAC, SC and BA for possible protective effects against the above mentioned toxicities induced by PCP and its metabolites. NAC did not seem to increase overall cell viability in cells exposed to the organochlorine compounds. BA and SC significantly increased cell viability in cells exposed to higher concentrations of PCP. This increase in cell viability may be attributed to the corresponding decrease in CYP1A1 activity and ROS generation in cells pre-treated with SC and BA prior to PCP exposure. Both plant extracts caused beneficial increases in viability in cells exposed to both TCHQ and TCBQ. These increases were more evident in cells treated with SC. Furthermore, SC and BA reduced increases in CYP1A1 activity caused by these metabolites and decreased ROS generation, more extensively than the hepatoprotective agent, NAC. However, both plants caused further mitochondrial depolarization and necrosis. There was however no corresponding decrease in viability which would be suspected with an increase in necrosis. Further studies are therefore

vital in order to make a definite conclusion concerning the protective effects of these extracts, with regards to PCP, TCHQ and TCBQ toxicity.

5.1 Limitations to the study and future work

Even though new insight into the mechanism of PCP, TCHQ and TCBQ was gained in the present study, a limitation is that no definite conclusion can be made as to what mode of cell death is induced by PCP and its metabolites. Further investigation is needed in this regard. In the future, regression analysis could also be performed in order to determine which of the hepatotoxic assays run in the present study is the best predictor of toxicity. The results of the present study can also be compared to *in vivo* studies in order to determine the accuracy of the model for *in vivo* extrapolation. Another limitations to the present study is that although the plant extracts and NAC seemed to possess some hepatoprotective properties, further investigation is needed to not only make a definite conclusion in this regard but also to elucidate the mechanism in which these substances induce their hepatoprotective effects.

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Annexure A – Ethics Approval



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

DATE: 27/05/2010

TO:

Prof V Steenkamp
Dept of Pharmacology

Best Prof V Steenkamp

RE.: Commercial Lines: The use of Commercial lines ~ Ms Ilka Schroeder

During the meeting held on 25/05/2011, the use of Commercial Lines were discussed.

The Faculty of Health Science Ethics Committee approved the use of the cell lines for the various assays.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Annexure B - Chemicals

For cell maintenance the following chemicals were purchased from Sigma Aldrich, Darmstadt Germany: Eagles Minimum Essential Medium (EMEM); Penicilin; Streptomycin. Fetal Calf Serum was purchased from Highveld Biological, JHB.

PCP was purchased from Chem Services whilst Tetrachloro-1,2-hydroquinone and Tetrachloro-1,4-benzoquinone were acquired from Dr Ehren Storfer GmbH, Ausburg, Germany. NAC was purchased from Sigma Aldrich, Darmstadt Germany.

The following positive controls were purchased from Sigma Aldrich, Darmstadt Germany: Tamoxifen; Omeprazole; 2,2'-azobis-2-methylpropanimidamidedihydrochloride (AAPH); Triton X-100 and Staurosporine.

For mechanistic assays the following chemicals were purchased from Sigma Aldrich, Darmstadt Germany: Neutral Red, 7-Ethoxy resorufin, propidium iodide (PI); Dichlorofluorosceindiacetate (DCFDA); 5,58,6,68-tetrachloro-1,18,3,38-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); ethylenediamine-tetraacetic acid (EDTA); phenylmethanesulfonylfluoride (PMSF) and 2-mecaptoethanol. Ethanol and Dimethyl Sulphoxide (DMSO) were purchased from Merck, whilst sodium chloride (NaCl) and acetic acid were purchased from Saarchem, RSA. Phosphate buffered saline was purchased from Becton, Dickinson and Company, USA.

Annexure C – Equipment

A Beckman-Coulter Allegra X22 centrifuge and a Reichert-Jung Microscope was used during cell plating. Plant material was ground with an Ika Analytical Mill. An ECX 800 universal plate reader was used for spectrophotometrical readings whilst a FluoStar Optima Fluorescent plate reader was used for fluorometric readings.

Annexure D – Conference outputs

- IE Schroeder, JJ van Tonder, V Steenkamp Effect of Pentachlorophenol and its metabolites on cell viability and CYP1A1 metabolism. University of Pretoria, Faculty of Health Sciences, Research Day, 31 August 2011 (Oral Presentation).
- IE Schroeder, JJ van Tonder, V Steenkamp Effect of Pentachlorophenol and its metabolites on cell viability and CYP1A1 metabolism. 6th International Conference on Pharmaceutical and Pharmacological Sciences, Durban, South Africa, 25-27 September 2011 (Oral Presentation).