

Mechanism of *Impila* (*Callilepis laureola*)-induced cytotoxicity in Hep G2 cells

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Abstract

Objectives: To determine the mechanism(s) of *Impila* (*Callilepis laureola*)-induced toxicity in human hepatoblastoma Hep G2 cells *in vitro* and the possible prevention of this toxicity by *N*-acetylcysteine (NAC).

Design and methods: Cells were treated with an aqueous extract of *Impila* (10 mg/mL) for up to 24 h. NAC (5 mM) was administered either concomitantly with *Impila* or one hour post *Impila* treatment. Cytotoxicity was quantitated spectrophotometrically by the metabolism of the tetrazolium dye MTT. Total glutathione (GSH) was measured using the Tietze assay.

Results: *Impila* produced cytotoxicity and depleted GSH in a concentration- and time-dependent manner. A significant depletion in GSH was observed after 15 min ($p < 0.0001$ vs. control), whereas significant cytotoxicity was only observed after at least 3 h ($p < 0.0001$ vs. control). Both concomitant and posttreatment with NAC prevented *Impila*-induced GSH depletion and resulted in a significant decrease in *Impila*-induced cytotoxicity ($p < 0.001$ vs. NAC-untreated cells).

Conclusion: Our results suggest the mechanism of *Impila*-induced cytotoxicity in Hep G2 cells *in vitro* involves depletion of cellular GSH. Preventing GSH depletion by supplementing cells with NAC reduces cytotoxicity. © 2002 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: *Callilepis laureola*; *Impila*; Traditional herbal medicines; Hepatotoxicity; Hep G2 cells; Glutathione; *N*-acetylcysteine

1. Introduction

The root of *Callilepis laureola* D.C. ("*Impila*" in Zulu) is widely used as a traditional herbal medicine in South Africa. *Impila* appears to serve as a multifunctional remedy and has been used to treat stomach complaints, cough, tapeworm infestations and impotence [1,2] and for cultural reasons [3]. Although it is unlikely that any accurate figures can be provided for the mortality due to traditional medicines poisonings in South Africa, the deaths from herbal poisonings in a regional hospital have decreased from 6 deaths per year in 1981–1985 to 1 death per year [4]. Some cases of fatal poisoning due to the ingestion of *Impila* have been documented [1,5,6]. *Impila* causes centrilobular liver necrosis, often accompanied with renal tubular necrosis in

humans and experimental animals [7]. The renal toxicity has been attributed to atractyloside (ATR), a diterpenoid glycoside that occurs naturally in several plants including *Impila* [8]. ATR is a well-established inhibitor of oxidative phosphorylation, however its involvement in *Impila*-induced hepatotoxicity remains uncertain. It has been suggested that the liver damage may result from a combined effect of more than one toxin or metabolite [5,7]. The cellular mechanism(s) involved in *Impila*-induced toxicity to the hepatocyte is still not known. We have previously reported the dose- and time-dependent cytotoxic effects of an aqueous extract of *Impila* in human liver-derived Hep G2 cells *in vitro* [9]. In this study, we used the same cell line to investigate further the mechanism of hepatocyte damage. We demonstrated that Hep G2 cells which have been maintained in our laboratory, express CYP 1A1, CYP 1A2 (implicating Ah receptor transcription), CYP 3A5 and CYP 2E1 but not CYP 2A1 and CYP 2C6 [10]. The cells retain cytochrome P450 activities inducible by aromatic hydrocar-

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bons (benz-antracene and di-benz-antracene) as well as by ethanol [11,12] and acetaminophen [13] but not phenobarbital. Hep G2 express 4 pmol/10⁶ cells of CYP 1A1, 2.5 pmol/10⁶ cells of CYP 1A2; 6 pmol/10⁶ cells of CYP 2E1 and 1 pmol/10⁶ cells of CYP 3A5 [12]. We tested the cytotoxic effects of *Impila* in relationship to oxidative stress by examining the effect of *Impila* on levels of GSH. We then examined the potential protective effects of NAC, a precursor to GSH, against *Impila*-induced cytotoxicity. Given the debate over the role of ATR in *Impila*-induced toxicity, we also investigated the potential toxic effects of pure ATR on cytotoxicity and GSH content in Hep G2 cells.

2. Methods

2.1. Materials

Bovine serum albumin (BSA), L-buthionine-(S,R)-sulfoximine (BSO), *N*-acetylcysteine, MTT (formazan 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), atryctyloside potassium salt (ATR), GSH reductase, GSH standard, NADPH (reduced nicotinamide adenine dinucleotide), 5-sulfosalicylic acid (SSA), EDTA (ethylenediaminetetraacetic acid), and DTNB (5,5-dithiobis-2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Minimal essential medium (α -MEM) was obtained from Gibco (Burlington, Ontario, Canada). Trypsin was purchased from Difco (Detroit, MI, USA) and was prepared as a 1% solution. The kit for protein determination was obtained from Bio-Rad Laboratories (Richmond, CA, USA). PBS (phosphate buffered saline without Ca²⁺ or Mg²⁺, pH 7.4) was used to wash cells and to remove medium. All plastic ware for cell cultures was obtained from Falcon (Becton Dickinson, Oxnard, CA, USA). All of the remaining reagents were of analytical grade, obtained from Sigma Chemical Co.

2.2. Preparation of *Impila* extract

Fresh roots from *Impila* were obtained from one of the investigators (S. Thomson) during the summer harvest and ground into a fine powder using a rotary grinder plate. The powder was dried slowly by warm convection fan over 12 h to avoid excessive thermal changes. An aqueous extract of *Impila* was prepared as previously described [9], using a method that most closely resembles that of traditional Zulu healers. The extract was prepared at a stock concentration of 60 mg dried powder per mL of distilled water, and boiled in a water bath for 1 h. The extract was then centrifuged at 2500 rpm for 10 min at room temperature. The supernatant was filter-sterilized under aseptic conditions and serial dilutions were made in plain α -MEM. Fresh extracts were prepared before each experiment.

2.3. Hep G2 cell line

Initially, Hep G2 cells were obtained from Wistar Institute (Philadelphia, PA, USA). The cells are in continuing use in our laboratory for the last 10 yr. Cells were seeded in collagen-coated Falcon flasks (1 × 10⁶ cells/mL) [10]. The cell counts were monitored using a Coulter counter (Coulter Electronics Inc., Hialeah, FL, USA). Cells were grown in α -MEM supplemented with 10% v/v heat inactivated fetal calf serum and maintained in a humidified atmosphere of 95% O₂ – 5% CO₂ at 37°C. The pH of the media was maintained at 7.4. At 70 to 80% confluence, cells were trypsinized and plated in 96-well round-bottom tissue culture plates to test cytotoxicity (Costar 3696, Cambridge, MA, USA). At the beginning of the experiment, when plated cells had reached 70 to 80% confluence, the growth medium was removed from the wells, cells were washed with PBS and fresh serum-free medium was used as base for treatment with *Impila*.

2.4. Experimental design

To determine if *Impila* has a concentration-dependent effect on GSH levels, cells were exposed for 6 h to either α -MEM (control) or to increasing concentrations of *Impila* (0.5–14 mg/mL). To determine the effects of *Impila* over time, cells were incubated with either α -MEM (control) or with *Impila* (10 mg/mL) for 15 min to 6 h, after which cytotoxicity and GSH content were measured in matched experiments. To determine if concomitant treatment with NAC could prevent *Impila*-induced cytotoxicity and GSH depletion, cells were treated with either plain α -MEM or with α -MEM supplemented with 5 mM NAC immediately before treatment with *Impila* (10 mg/mL). Cells were then incubated for 1, 3, 6 or 24 h, after which cytotoxicity and GSH content were determined in matched experiments. To determine if posttreatment with NAC could prevent *Impila*-induced toxicity, cells were treated with *Impila* (10 mg/mL) for 3, 6 or 24 h. One hour following treatment with the *Impila* extract, cells were supplemented with 5 mM NAC. Cytotoxicity and GSH content were then measured as in the previous experiment. The next set of experiments aimed to determine the potential toxic effects of pure ATR. ATR was first dissolved in DMSO and then diluted in α -MEM such that cells were exposed to nontoxic DMSO concentrations [11]. Hep G2 cells were exposed to either the appropriate vehicle (control) or to increasing concentrations of ATR (5 μ M to 5 mM) and incubated at 37°C (95% O₂ – 5% CO₂) for a period of 24 h. An additional experiment was set up to show the role of BSO in blocking the GSH synthesis. We pretreated Hep G2 cells for 2 h with 2 mM BSO as previously described [12]. After the 2 h exposure to 2 mM BSO the cells were exposed 24 h to *Impila* extract in the presence or absence of a GSH precursor S-Adenosyl-methionine (SAMe).

Cytotoxicity and GSH content were then measured as

described for previous experiments. For each experiment, all components were filter sterilized and the entire procedure was conducted under aseptic conditions.

2.5. Cytotoxicity assay

Cytotoxicity was assayed using the MTT test [12]. We have previously shown that the tetrazolium dye MTT is an adequate indicator of cell viability in human hepatocyte systems intoxicated *in vitro* with acetaminophen [13] and ethanol [10]. The methods of Mosmann and Carmichael [14] were used with our modifications [10] for cells grown directly in the 96-well plate. MTT (100 μ L of a 1 mg/mL solution) was added to each well of the 96-well plate and incubated for 1 h at 37°C, protected from light. At the end of the incubation, the untransformed MTT was removed from the well by aspiration and 100 μ L of isopropyl alcohol was added to each well. The plate was then shaken vigorously (Microshaker II Dynatech, Dyna-Med, Toronto, Ontario, Canada) at speed setting 10 s/min to ensure that the blue formazan was fully solubilized. The optical density of each well was measured at dual wavelength mode (595 nm and 655 nm) using an automatic multiwell microplate spectrophotometer (Maxline Microplate Reader, Molecular Device Corp., Menlo Park, CA, USA). Cytoviability of control cells was considered to be 100% (0% cytotoxicity). For the treated cells viability was expressed as a percentage of control cells. All determinations were carried out in replicates of twelve (6 wells/plate \times 2 plates).

2.6. Glutathione assay

Total cellular GSH was determined using a slight variation of the Tietze assay [15] as modified by Cribb et al. [16]. The Tietze assay is a sensitive and specific method for the determination of both reduced (GSH) and oxidized (GSSG) forms of glutathione in unknown samples [17]. A stock buffer of 0.1 mM sodium phosphate, pH 7.5, with 1 mM EDTA was made up in distilled water and used to prepare separate solutions of 12 mM NADPH, 0.1 mM DTNB and 50 U/mL GSH reductase. The assay was carried out directly in the 96-well plates. Immediately before the assay, cells were lysed by adding 25 μ L of 2% SSA to each well and shaking plates vigorously at speed setting 10 s/min. To each well was then added 100 μ L DTNB, 20 μ L GSH reductase, and 20 μ L NADPH. Absorbance at 415 nm with a reference wavelength of 595 nm was measured every 30 s for 1.5 min at room temperature using an automatic multiwell microplate spectrophotometer (Maxline Microplate Reader, Molecular Device Corp., Menlo Park, CA, USA), and final values were calculated as a mean of the three readings. The automatic mix function on the microplate reader was used to shake the plate before each reading. Standards of known GSH content were made up by serial dilution in 2% SSA and used to construct a standard curve. The rate of increase in absorbance at each concentration of GSH standard was

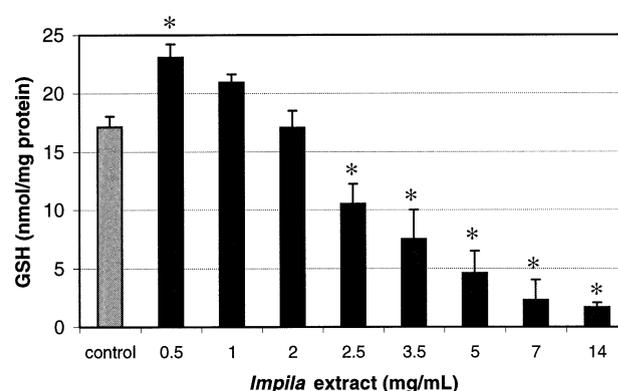


Fig. 1. Dose-dependent depletion of total GSH induced by *Impila* extract in HepG2 cells *in vitro*. Hepatocytes plated directly in 96-well trays were exposed for 6 h to plain medium (control) or to an aqueous extract of *Impila*. GSH content was measured using the Tietze assay. Treatment with the lowest concentration of *Impila* (0.5 mg/mL) resulted in an increase in GSH levels compared to control, however higher concentrations of *Impila* resulted in GSH depletion (* $p < 0.0001$ vs. control). GSH levels after treatment with 1 mg/mL and 2 mg/mL *Impila* were not significantly different from the control. Each bar represents the mean \pm SD of 12 wells (6 wells/plate \times 2 plates).

linear over the 1.5 min assay. Total protein concentration for each sample was determined with a Bio-Rad kit [18] using bovine serum albumin as standard, such that GSH levels could be expressed as nmol per mg total protein. All determinations were carried out in multiples of twelve (6 wells/plate \times 2 plates).

2.7. Statistical analysis

All data are expressed as means \pm standard deviation (SD). The results were compared using a one-way analysis of variance (ANOVA) with a Bonferroni correction for multiple comparisons. Differences between NAC-untreated and NAC-treated cells were analyzed using the Student's *t*-test. A *p* value < 0.05 was considered significant. Statistical analysis was conducted using SPSS 9.0 for Windows (SPSS Inc. Chicago, IL, USA).

3. Results

A 6 h incubation with *Impila* caused a dose-dependent depletion of total cellular GSH in Hep G2 cells (Fig. 1). Treatment with the lowest concentration of *Impila* (0.5 mg/mL) actually produced an increase in GSH levels compared to control ($p < 0.0001$). Higher concentrations, however, caused significant depletion of GSH ($p < 0.0001$ vs. control at 2.5–4 mg/mL).

The next experiment was designed to study the effects of *Impila* on cytotoxicity and GSH levels over a 6 h time frame. In this experiment, a concentration of 10 mg/mL *Impila* was used and cytotoxicity and GSH levels were determined after each time point in matched 96-well plates.

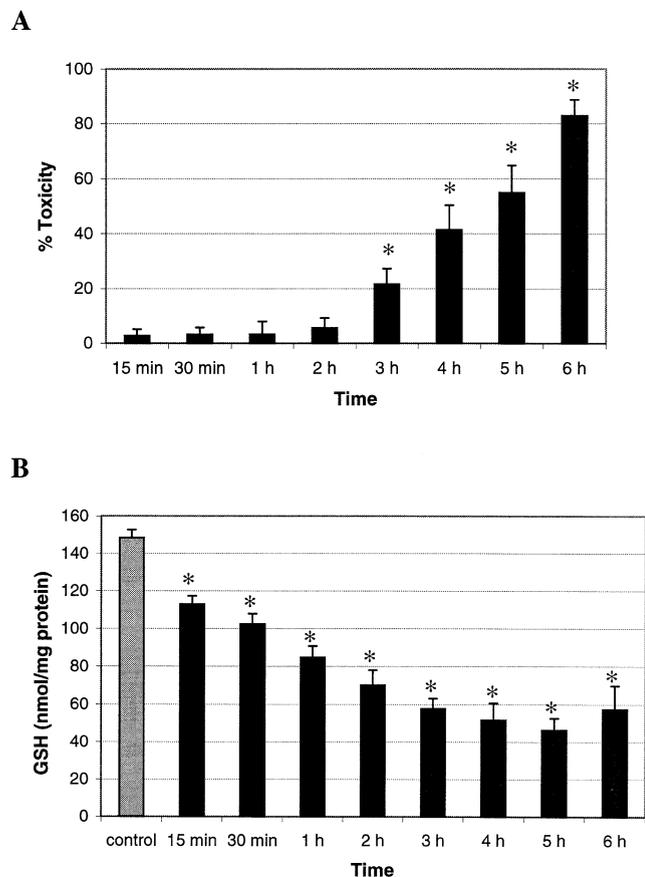


Fig. 2. (A) Time course of the cytotoxic effect of *Impila* extract in Hep G2 cells. Hepatocytes plated directly in 96-well trays were exposed for 15 min to plain medium (control) or to an aqueous extract of *Impila* (10 mg/mL). Cytotoxicity was assessed by the MTT test and expressed as a percentage of the control. Cytotoxicity was significant at 3, 4, 5 and 6 h (* $p < 0.0001$ vs. control). (B) Time course of the effect of *Impila* on total cellular GSH content determined in matched 96-well plates. GSH content was measured using the Tietze assay. A significant depletion in GSH was observed at all time points (* $p < 0.0001$ vs. control). No significant difference in GSH content was found between 3, 4, 5 and 6 h, thus *Impila*-induced GSH depletion was maximal at 3 h. Each bar represents the mean \pm SD of 12 wells (6 wells/plate \times 2 plates).

Both cytotoxicity and GSH depletion were found to increase over time (Fig. 2). Significant GSH depletion, however, was found to occur before any significant cytotoxic effects. After 15 min, *Impila* significantly decreased GSH levels by 24% compared to control (Fig. 2B). Maximal depletion of GSH occurred after 3 h (61% decrease from control, $p < 0.0001$; no significant differences in GSH content were found between 3, 4, 5 and 6 h). Significant cytotoxicity was observed only after at least 3 h of *Impila* treatment (22% toxicity, $p < 0.0001$ vs. control, Fig. 2A). Cytotoxicity increased over 4, 5 and 6 h, with over 80% cytotoxicity occurring after 6 h of treatment.

Next we wanted to determine whether NAC could prevent *Impila*-induced cytotoxicity and GSH depletion. In initial experiments, cells were supplemented with 0.5 mM NAC immediately before treatment with *Impila* (10 mg/mL). NAC caused a slight reduction in cytotoxicity after a

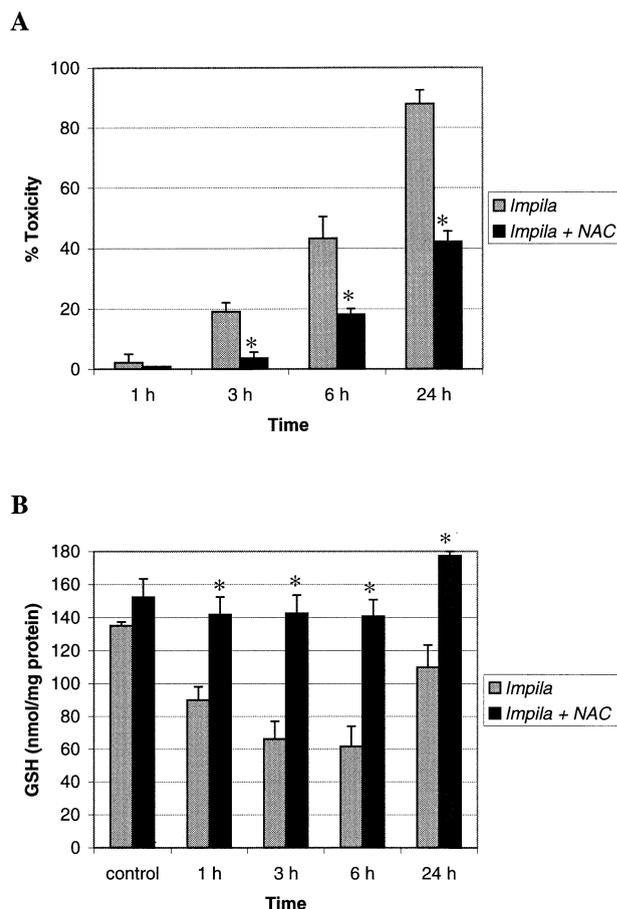


Fig. 3. Concomitant treatment with NAC prevents *Impila*-induced cytotoxicity and GSH depletion in Hep G2 cells. (A) Hepatocytes plated directly in 96-well trays were exposed for 1 to 24 h to plain medium (control) or to an aqueous extract of *Impila* (10 mg/mL). NAC-treated cells received 5 mM NAC directly before exposure to *Impila* extract. Cytotoxicity was assessed by the MTT test and expressed as a percentage of the control. NAC treatment resulted in a significant decrease in *Impila*-induced cytotoxicity compared to NAC-untreated cells (* $p < 0.01$ at 3, 6 and 24 h). (B) Total cellular GSH content was measured in matched 96-well plates using the Tietze assay. NAC prevented *Impila*-induced GSH depletion and maintained the cellular GSH content at control values. Total GSH content was significantly higher in cells concomitantly treated with NAC compared to NAC-untreated cells at all time points (* $p < 0.01$). Each bar represents the mean \pm SD of 12 wells (6 wells/plate \times 2 plates).

6 h treatment with *Impila* ($47.2 \pm 7.9\%$ toxicity in NAC-untreated vs. $34.7 \pm 3.2\%$ toxicity in NAC-treated cells, $p < 0.01$). In matched experiments, however, 0.5 mM NAC treatment failed to prevent *Impila*-induced GSH reduction (47% decrease from control in NAC-untreated vs. 44% decrease from control in NAC-treated cells, $p = 0.27$). In the next set of experiments, cells were supplemented with a higher concentration of NAC (5 mM) immediately before treatment with *Impila* (10 mg/mL). NAC treatment resulted in a significant decrease in cytotoxicity after 3, 6 and 24 h of *Impila* treatment ($43.3 \pm 7.2\%$ toxicity in NAC-untreated vs. $18.1 \pm 1.9\%$ toxicity in NAC-treated cells at 6 h, $p < 0.001$, Fig. 3A). In matched experiments, 5 mM NAC com-

pletely abolished *Impila*-induced GSH depletion and maintained the cellular GSH content at control values (Fig. 3B).

Next we wanted to determine whether NAC could still have protective effects when given to cells 1 h after they had been treated with *Impila*. NAC (5 mM) resulted in a significant decrease in cytotoxicity after 6 and 24 h of *Impila* treatment (Table 1). Again, 5 mM NAC completely abolished *Impila*-induced GSH depletion and maintained levels at control values.

An additional experiment was set up to show the role of BSO in blocking the GSH synthesis. Pretreated Hep G2 cells for 2 h with 2 mM BSO as presented 23% toxicity vs. the controls. After the 2 h of BSO exposure and additional exposure 24 h to *Impila* extract (10 mg/mL), cells presented an additional 15% toxicity ($p < 0.001$). A total of 38% toxicity vs. control was measured ($p < 0.0001$). When cells were exposed to *Impila* (10 mg/mL) in the presence of SAME the toxicity did not increase further vs. the previous value.

Impila (10 mg/mL) did not have a significant effect on the total protein concentration in Hep G2 cells at 1, 3 and 6 h incubation periods (no significant difference from control, data not shown). A 24 h incubation with *Impila*, however, caused a significant decrease in total protein concentration (22.8% decrease from control, $p < 0.0001$). This decrease was prevented by both concomitant and post-NAC treatment (5 mM).

In marked contrast to *Impila*, 24 h incubation with pure ATR had no significant effect on cytotoxicity at any concentration tested. Additionally, in matched experiments, pure ATR had no effect on the total intracellular GSH content. GSH content in ATR-treated cells was not significantly different from the control value at all concentrations tested.

The GSH content in the cells pretreated with BSO was significantly lower (60 nmol/mg protein) ($p < 0.05$) vs. the control values (96 nmol/mg protein). *Impila* extract exposure for 24 h., further reduced the GSH content to 50 nmol/mg protein, where a concomitant treatment with *Impila* and SAME resulted in 68 nmol/mg protein.

4. Discussion

We have previously reviewed the toxicity of *Impila* and reported preliminary results showing a concentration- and time-dependent cytotoxic effect of an aqueous extract of *Impila* in Hep G2 cells *in vitro* [9]. The Hep G2 cell line offers a reliable *in vitro* system to study drug-induced hepatotoxicity [10,19]. The aim of the present study was to use this model to investigate whether the mechanism of *Impila*-induced cytotoxicity involves depletion of cellular GSH.

GSH is an important tripeptide that plays a vital role in the detoxification of exogenous and endogenous compounds, either by conjugating with an electrophilic drug or its toxic metabolites or by acting as a reducing agent in the

metabolism of peroxides and free radicals [20,21]. In the present study an aqueous extract of *Impila* depleted GSH levels in a concentration- and time-dependent manner. The initial increase in GSH content observed at a low concentration of *Impila* suggests the cell is responding to the chemical insult by preparing itself for defense by boosting its detoxifying capacity. At higher concentrations of *Impila*, however, GSH levels were decreased and cytotoxicity was observed [9]. GSH depletion preceded cell damage, where *Impila*-induced cytotoxicity occurred only after GSH levels had been depleted to a maximum. This observation suggests that the initial mechanism of *Impila* toxicity involves the depletion of GSH, after which subsequent events ultimately lead to cell death.

The stability of an aqueous extract of *Impila* over time has not yet been defined; therefore instead of preparing one stock solution for all experiments, we opted to prepare a fresh extract immediately before each individual experiment. Although the extraction process was always conducted under similar conditions using a precise protocol, it is possible that the concentration of toxic constituents varied between different extract preparations. This may explain why an *Impila* extract (10 mg/mL) produced nearly 80% toxicity after 6 h in initial experiments (Fig. 2A), yet produced only 40% toxicity in subsequent experiments (Fig. 3A).

A high concentration of GSH is found in eukaryotic cells (2–10 mM), 90% of which is located in the cytosol, 10% in the mitochondria, and a small percentage in the endoplasmic reticulum [22–24]. Evidence suggests that severe GSH depletion to about 20 to 30% of total intracellular levels can impair the cell's defense against toxic compounds and may result in cell injury and death [11,25,26]. In the case of acetaminophen intoxication, it has been estimated that more than 70% of glutathione must be lost before cell damage occurs [22,27]. In our study, the *Impila* extract at a concentration of 10 mg/mL did not decrease the total cellular GSH content below approximately 38% of the control level, however cytotoxicity at this point was over 80%. One possible explanation for the high toxicity observed may be due to severe depletion of the mitochondrial GSH pool. The Tietze assay used in our study measures total cellular GSH, and thus does not differentiate between cytosolic and mitochondrial pools. The mitochondrial pool of GSH, however, appears to be specifically important in cell defense and the maintenance of vital cell functions [26,28–30]. Studies have shown that mitochondrial GSH levels appear to be more closely linked to cell damage than do cytosolic GSH levels [21]. For example, in a study using isolated hepatocytes, Meredith and Reed [23,31] showed that chemical-induced cytotoxicity correlated with the depletion of mitochondrial GSH, whereas depletion of cytosolic GSH had no effect on cell viability. Therefore, although the *Impila* extract (10 mg/mL) did not cause total GSH levels to drop below approximately 38% of control levels, it is possible that the mitochondrial pool of GSH was depleted beyond the thresh-

old for danger to the hepatocyte. Thus, further experiments investigating *Impila*-induced GSH depletion of both mitochondrial and cytosolic pools are merited. Additionally, Hep G2 cells contain only about 50% of the intracellular GSH content of freshly isolated human hepatocytes [20,32]; therefore they may be more susceptible to *Impila*-induced cytotoxicity than normal hepatocytes.

While cellular GSH levels decreased over 1 to 6 h of treatment with the *Impila* extract, an increase in GSH was observed at 24 h compared to the level observed at 6 h. It is possible that the surviving hepatocytes have adapted to the toxic environment by increasing their production of GSH as a protective strategy. The apparent increase in GSH content at 24 h may also, in part, be due to the high level of *Impila*-induced cytotoxicity, which resulted in a decrease in the amount of total protein measured. GSH values were expressed per mg of total protein, thus the GSH content at 24 h may be slightly inflated since *Impila* caused a decrease in the overall total protein content at this time point as well, whereas protein content at 1 to 6 h was not significantly different from that of control values.

N-acetylcysteine (NAC) increases GSH synthesis under conditions of depleted levels by supplying a source of thiols to the cell [21,33]. NAC has long been used as an important antidote in clinical cases of acetaminophen intoxication [34–36]. *In vitro* studies have shown that NAC prevents depletion of GSH in acetaminophen-treated hepatocytes [37–39], and also offers cytoprotective effects against other toxins that deplete cellular GSH [40]. In our study, NAC (5 mM) reduced *Impila*-induced toxicity by over 50%. The protection of NAC against *Impila*-induced cytotoxicity highlights the significance of GSH-depletion as an early event in the mechanism of *Impila*-induced cell death. NAC had cytoprotective effects when given either concomitantly with *Impila* or one hour after *Impila* treatment. This observation demonstrates that regardless of the significant depletion of GSH at 1 h of *Impila* treatment, recovery is still possible with NAC supplementation and that at this time point irreversible cell damage has not yet occurred. Despite the addition of NAC, however, *Impila* still produced some toxicity. This suggests that another mechanism, independent of GSH, may also be involved in *Impila*-induced toxicity. Alternatively, the concentration of *Impila* may be so high that the GSH defense system, even with added NAC, is still not sufficient to protect the hepatocyte from toxic damage. To bring additional light in the role of GSH in *Impila*-induced toxicity and its prevention we introduce a new experiment by which we blocked the GSH synthesis by pretreating the cells to a GSH blocker BSO [20]. In the GSH depleted cells addition of *Impila* further-induce toxicity by continually depleting GSH. The mechanism can be reverse by the concomitant exposure of Hep G2 to the plant extract and a precursor of GSH (SAME).

Given our results, the following hypothesis could be proposed. One or several constituents of *Impila* may be biotransformed in the hepatocyte to an electrophilic me-

tabolite(s) or cause the formation of reactive oxygen species (ROS), thereby inducing oxidative stress. The metabolite(s) or ROS is detoxified by GSH, however at higher concentrations of *Impila* this detoxification system becomes overwhelmed. The subsequent depletion of GSH renders the hepatocyte vulnerable to further attack by the reactive metabolite(s) leading to cell damage and ultimate cell death. The toxic events that follow GSH depletion require further investigation. Studies have shown that ATR, one of the toxic constituents that has been isolated from *Impila*, may undergo transformation in the liver resulting in the production of active radicals leading to a toxic effect similar to that observed with acetaminophen poisoning [41]. Other evidence suggests that ATR may trigger apoptosis by inducing opening of the mitochondrial transition pore, cytochrome c release and caspase activation [42–46]. Preliminary studies using HuH-1 human hepatoma cells have shown that ATR and extracts of *Impila* give rise to severe derangement of mitochondrial morphology, destruction of the cytosolic tubulin network, and inhibition of cell division, however the effects caused by ATR were reported to differ to those caused by *Impila* [7].

Interestingly, in the present study, pure ATR had no effect on the cytoviability or the GSH content of Hep G2 cells at any concentration tested. This finding raises several important points of discussion in regards to the debate over the involvement of ATR in *Impila*-induced hepatotoxicity. Firstly, our observed lack of toxicity of ATR is in agreement with the *in vivo* work conducted by Wainwright et al. [5] and Bhoola [47]. These investigators showed that an *Impila* extract produced hepatic and renal necrosis in rats, however when rats were treated with a purified form of ATR that had been isolated from the *Impila* extract, the hepatotoxic action was lost. Our results thus support the previous suggestion that *Impila* contains another component, other than ATR, that is responsible for the plant's hepatotoxic effects [5]. In contrast to our findings, however, *in vitro* studies conducted by Obatomi et al. [48,49] showed that ATR (0.2 mM to 2 mM) caused a concentration-dependent depletion of intracellular GSH in pig and rat kidney and liver slices. However, the authors also reported that while ATR (≥ 1 mM) caused cytotoxicity in kidney slices, no cytotoxicity was observed in liver slices, as measured by the MTT test. ATR (≥ 0.5 mM) did cause some lactate dehydrogenase leakage from pig liver slices, indicating that some cytotoxic damage was occurring. Furthermore, ATR caused an increase in lipid peroxidation and the GSSG:GSH ratio in liver slices from both species, but not in kidney slices [48–50]. Based on these findings, the authors suggested that mechanistic differences exist in ATR-induced toxicity in the liver and kidney, in which the liver toxicity results from oxidative stress, which may involve an ATR metabolite and/or the formation of free radicals [48,49]. No data have been subsequently published in regards to the role of metabolism in

ATR-induced toxicity, or the enzymes that may be involved in its potential biotransformation.

If the toxicity of ATR is dependent on metabolic activation, it is possible that the Hep G2 cell line used in the present study does not express the metabolizing enzyme or display adequate enzyme activity to biotransform ATR into its toxic intermediate, which may explain why we did not observe any ATR-induced cytotoxicity. However, our finding that the crude *Impila* extract did produce significant cytotoxicity and GSH depletion in Hep G2 cells, strongly suggests that *Impila* contains a hepatotoxic component(s) independent of ATR or an ATR metabolite. In contrast to pure ATR, this other component causes a marked depletion of GSH in Hep G2 cells, which ultimately results in cytotoxicity.

In conclusion, our results suggest: (1) GSH depletion is an early and critical event in the mechanism of *Impila*-induced cytotoxicity in Hep G2 cells *in vitro*; (2) preventing GSH depletion by supplementing cells with NAC or SAME can significantly reduce the cytotoxic effects of *Impila*; and (3) *Impila*-induced GSH depletion and cytotoxicity in Hep G2 cells does not result from the effects of ATR alone. More work is needed to identify the other toxic constituents of *Impila*. Additionally, the potential role of NAC or SAME as antidote in clinical cases of *Impila*-poisoning deserves further investigation.

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