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The toxicity of *Callilepis laureola*, a South African traditional herbal medicine

Alpa Popat[a,b], Neil H. Shear[a,b], Izabella Malkiewicz[a], Michael J. Stewart[c], Vanessa Steenkamp[c], Stuart Thomson[d], Manuela G. Neuman[a,b,*]

[a]*Division of Clinical Pharmacology, E240, Sunnybrook and Women's College Health Sciences Centre, 2075 Bayview Avenue, Toronto, Ontario, Canada*

[b]*Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada*

[c]*Toxicology Unit, Department of Chemical Pathology, South African Institute for Medical Research, University of the Witwatersrand, Witwatersrand, South Africa*

[d]*Gaia Research Institute, Knysna, South Africa*

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Abstract

Objectives: To review the literature on the toxicity of *Callilepis laureola*, and to assess the cytotoxicity of *C. laureola* in human hepatoblastoma Hep G2 cells *in vitro.*

Design and methods: Cells were incubated for up to 48 h in the presence of increasing concentrations of an aqueous extract of *C. laureola* (0.3–13.3 mg/mL). Cytotoxicity was quantitated spectrophotometrically by the metabolism of the tetrazolium dye MTT. Cytoviability of the control cells was considered to be 100%.

Results: *C. laureola* produced cytotoxicity in a concentration-dependent manner. Cytotoxicity was significant at all concentrations tested (0.3–2.5 mg/mL, $p < 0.05$ vs. controls and 3.3–13.3 mg/mL, $p < 0.0001$ vs. controls). After 6 h, 100% toxicity was observed at a concentration of 6.7 mg/mL.

Conclusion: *C. laureola* causes significant cytotoxicity in Hep G2 cells *in vitro.* These findings are in accordance with the observed hepatotoxicity in clinical cases of *C. laureola* poisoning.

Keywords: Callilepis laureola; Impila; African; Traditional herbal medicines; Hep G2 cells; Hepatotoxicity

1. Introduction

Within the past two decades, the use of medicinal plants has increased significantly in developed countries [1–3]. It is estimated that 40% of the adult American population use herbal remedies [4]; similar trends are also occurring in Canada [5,6], Europe [7], and Australia [8]. There is a mythical yet predominant view that herbal medicines are harmless and free of side effects because they are "natural" [7,9]. There have been several cases, however, of hepatic injury and even death associated with their use [10,11]. The safety of several commercially available herbs has recently come into question due to reports of adverse reactions and potential interactions with prescription drugs [10,12–15]. The effective and safe use of medicinal herbs has therefore been identified as a top research priority; and the implementation of regulatory procedures and investigations on safety are currently underway in developed countries [5].

While not addressed as frequently in the literature, the safety of herbal medicines used in underdeveloped countries is also a major concern. In South Africa, it is estimated that between 60 to 85% of the native population use traditional medicines, usually in combinations [16,17]. Cases of acute poisoning due to traditional medicines are not uncommon, many of which have resulted in significant morbidity and mortality [18], with mortality estimated to be as high as 10,000 to 20,000 per annum [19]. Joubert and Sebata [20] analyzed 277 cases of acute poisoning admitted to Ga-Rankuwa Hospital, Pretoria over a 12 month period [1981–1982] and found that 18% were due to ingestion of traditional medicines; 26% of these cases resulted in death. In a continuing study by Venter and Joubert [21], 1306 cases of

* Corresponding author.

E-mail address: manuela@sten.sunnybrook.utoronto.ca (M.G. Neuman).

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acute poisoning were examined at the same hospital over a 5 yr period [1981–1982]; 15.8% were due to traditional medicines; 15.3% of these cases resulted in death. Overall, the authors found that poisoning with traditional medicines resulted in the highest mortality, accounting for 51.7% of all deaths that were due to acute poisoning. In both studies, patients were predominantly male and the majority of admissions were children between the age of 1 to 5 yr. Traditional healers were the main source of the medicines, and in some cases substances were bought at a shop for African remedies [21]. The majority of poisonings were accidental, only 4% were due to deliberate self-poisoning. A third study by Stewart *et al.* analyzed the Johannesburg forensic database over 5 yr [1991–1995] and found that traditional remedies, all being of plant origin, were involved in 43% of poisoning cases [22].

While these studies have provided estimates, it is suspected that the true number of poisoning cases from traditional medicines is far greater [23]. Medically certified information on the mortality among native South Africans is lacking, especially for rural areas where deaths are not always registered [19]. Many poisoning cases are thought to remain undiagnosed since patients residing in rural areas may die before reaching a hospital [23]. Furthermore, autopsies are not routinely conducted, and the cause of death is not always determined or documented on the certificate [17], thus many poisoning cases may go unrecognized. Detection of traditional medicine poisoning is further complicated due to the lack of analytical techniques required to make a confident diagnosis [22,24]. Due to a shortage in resources, diagnostic tools are either limited or have not yet been developed. Moreover, the plant component of the traditional remedy responsible for the observed toxicity may not be known. In some cases, the culprit plant has been identified through direct questioning of the patient or the patient's family [17,25,26]. People are generally very reluctant to admit the use of herbal remedies, however, often because hospitals tend to hold a negative view toward traditional medicines [23,27], and also because of the cultural secrecy surrounding their use [18,28,29].

In the present study, we investigate the *in vitro* hepatotoxicity of one known toxic herb: *Callilepis laureola. C. laureola* is a traditional remedy commonly used by the Zulu who are predominantly located in the KwaZulu-Natal region in the northeast of South Africa [30]. *C. laureola,* a member of the family Compositae, is a herbaceous perennial plant found commonly in grassland habitats of eastern South Africa (*personal communications,* Geoff Nichols, Silverglen Medicinal Plant Nursery, Durban, August, 2000, Fig. 1). *C. laureola* is known to be "very poisonous and has even been responsible for several deaths among the Zulu" [31]. It has been estimated that the plant is responsible for up to 1500 deaths per annum in KwaZulu-Natal alone, one of nine provinces in South Africa [27,32]. The plant is commonly known as *Impila,* which ironically is the Zulu word for "health" [30].

Fig. 1. *Callilepis laureola (Impila).* The plant bears a tuberous root, similar to a potato [26,30,54] with a characteristic bulbous shape and pungent odour [22]. During the months of August to November, *C. laureola* yields solitary creamy white flowers with a purple disc. Photograph taken in the Northern KwaZulu-Natal region, courtesy of Geoff Nichols, Silverglen Medicinal Plant Nursery, Durban, South Africa.

Although there are no approved medical uses of *Impila* from a health regulatory standpoint, the plant is widely used among the Zulu and appears to serve as a multi-purpose remedy [23,31]. Reports indicate it is used to treat stomach problems [23], tape worm infestations [33], impotence [25], cough [33,34], and to induce fertility [35]. *Impila* is also administered to pregnant women by traditional birth attendants to "ensure the health of the mother and child" [36] and to facilitate labor [37]. A tonic made from the root is also taken by young girls in the early stages of menstruation [19,38]. The greatest and most valued attribute of this plant, however, appears to lie in its "protective powers" [30] in warding off "evil spirits" [23]. For example, an *Impila* decoction consumed before festivals is thought to offer protection from "those harboring evil intent" [26]. Parents who have lost previous children to illness may administer *Impila* enemas to their current children for the belief it will "protect" them. It is suspected that these magical beliefs are the primary reason for the common use of *Impila* in young children [39], and the high *Impila*-related mortality in children under the age of 5 yr [23].

Impila is most often prepared using the tuberous rootstock of the plant, while the leaves are reputed to have

minimal curative properties [31]. The tuber may be harvested and collected in the winter, and dried and crushed into a powder [30,31,40]. Alternatively, a fresh piece of the tuber, the size of a forefinger, may be chopped and bruised [26]. The resultant powder is boiled for approximately 30 min to 1 h in a suitable volume of water and the decoction is administered either orally or as an enema [23,30]. It has been estimated that each dose of the herbal remedy is prepared from approximately 10 g of plant material [32].

The danger of *C. laureola* was first documented in 1909 by A.T. Bryant [31]. Numerous cases of *Impila*-induced hepatic and renal toxicity emerged in the medical literature during the 1970s [17,25,26,39], and even received some media coverage [41]. A study by Wainwright and Schonland [17] conducted in the late 1970s describes the high incidence of centrilobular liver necrosis in the native African population of KwaZulu-Natal. *Impila* was identified as the primary causative agent [26,42], and since this time there have been regular reports of fatal *Impila* intoxications [18,23,30,39,43,44]. A comprehensive review of *Impila*-related poisonings in South Africa has been written by Bye and Dutton [30].

The toxicity of *Impila* appears to be very sudden in onset, and it is suspected that many patients do not reach a hospital before death [23]. Cases that have been documented indicate the duration of illness before hospital admission is less than 1 day in 40% of patients [17]. Generalized symptoms of intoxication include abdominal pain, vomiting, diarrhea, a disturbed level of consciousness, convulsions, and acute liver and renal failure leading to severe hypoglycemia and metabolic acidosis [17,23,25,37,39]. The fatalities due to *C. laureola* toxicity are significant. As reported by various investigators [17,23,42,44], it is estimated that 63% of patients die within 24 h, and a further 28% die within 5 days, thus bringing the total mortality to 91%. Autopsy findings in cases of *Impila* poisoning consistently show centrilobular zonal necrosis of the liver which is usually reduced in weight [17,23]. Other common findings include a pale and swollen renal cortex and congested medulla, and acute tubular necrosis of proximal convoluted tubules and loops of Henle. Hemorrhages are often present in the lungs, skin and intestine.

Despite its reputed toxicity, *Impila* continues to be a very popular and commonly used traditional remedy in South Africa [23,30,42]. *C. laureola* has been identified among the medicinal plants grown by traditional herbalists [45]. A survey conducted by Wainwright *et al.* on a randomized sample of residents of Umlazi township revealed that 30% used or had used *Impila* and people generally regarded it as a very valuable medicinal plant [26]. A second report by Ellis [40] estimated that the plant is used by at least 50% of the native population in Natal, making *Impila* the second most widely used traditional medicine in the area.

If the toxicity of *C. laureola* is so well established, why then is the plant still being used significantly in South Africa? There appears to be several complex answers to this question. Currently there is no legislation controlling traditional medicines in South Africa, and the regulatory standards and public education required to ensure their safe use have yet to be implemented [19,46]. In rural areas, traditional healers are the primary source for obtaining such medicines, whereas in towns and cities, traditional medicines are readily available in African medicine shops where they are sold over-the-counter. In an article by Varga and Veale [47], there is brief mention of *C. laureola* being banned by the KwaZulu-Natal Provincial Department of Health in 1995. The ban appeared to be a result of several chemical studies that demonstrated the plant's extreme toxicity [26,48–50]. In 1999, however, Steenkamp *et al.* report two cases of *C. laureola* poisoning in a mother and child, highlighting the point that *Impila* poisoning continues to be a recurring phenomenon in South Africa.

Another issue to consider is the cultural context in which traditional medicines are used. *Impila* is most commonly used for the magical properties it is believed to possess [30]. While a discussion on the African cultural beliefs of health and illness is far beyond the scope of this review, some points are worthy of mention to understand why such toxic herbs continue to be used. Spiritual beliefs are an integral component of the traditional African concepts of health and sickness [29,51], and while some illnesses are attributed to natural causes, others are thought to be the result of an "evil spell", or the consequence one must suffer for violating the ancestral spirits [36,45]. The respect for traditional healers and the belief in the curative properties of traditional medicines is so deep-rooted, that often a fatality resulting from a toxic herb will wrongfully be blamed on the underlying "illness" for which the herb was taken. Moreover, cases where poisoned children have died in a hospital setting have shown parents to be more suspicious of western medical practices, believing that the doctors and not the herb contributed to the child's death [30].

Other points of consideration are the factors that affect the toxicity of the herb itself. The level of pharmacologically active constituents found in plants is influenced by environmental conditions such as soil and climate. The toxicity of some plants has been shown to vary with season [27]. Additionally, a report by Seedat & Hitchcock [25] indicates that dosage may play a vital role in the toxicity of *Impila.* The authors report a case involving a 42 yr old male who developed hyperkalaemia and acute renal failure due to the ingestion of *C. laureola.* Upon questioning, the patient said that he was prescribed *Impila* by a traditional healer. The patient did not follow the healer's instructions of preparation, however, and took a dose that was at least 8 times greater than that prescribed. The lack of safety regulation and the ease at which herbal medicines may be obtained likely increase the occurrence of such fatal errors. Furthermore, while most traditional healers must undergo a rigorous and extensive apprenticeship before becoming a qualified healer, some may not possess adequate knowledge, skill

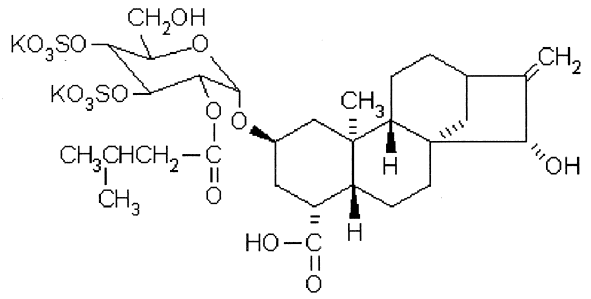

Fig. 2. Chemical structure of atractyloside.

or experience, and thus fatal errors may also be made on behalf of the prescriber [52].

A lack of knowledge among traditional healers, vendors and the public in regards to the appropriate use of traditional medicines is demonstrated in a correspondence by Bodenstein published in the South African Medical Journal [53]. The author quotes his discussion with a colleague about the strict "ancient rules" regarding the use of *Impila* as an herbal remedy: "*impila* is never given to a child under the age of 10; it is never given by way of an enema; it is never used in arbitrary doses nor in any but the weakest solution; when swallowed, it must never be allowed to be absorbed; in other words, it is used exclusively in the form of treatment known as *phalaza* (*i.e.*, swallowing a large volume of a weak decoction, followed by immediate inducement of complete or near-complete catharsis). If used with the same irresponsibility and ignorance, many of the finest and most celebrated Western remedies must surely be quite as deadly as *impila*" [53]. There is little doubt that a lack in knowledge and awareness of these strict rules has contributed to the numerous cases of *Impila*-induced fatalities.

Although clinical cases of *C. laureola*-induced toxicity are well documented in the literature, the mechanism by which the plant produces hepatic and renal toxicity is not completely understood. The two major toxic components extracted from the tuber of *C. laureola* are atractyloside (ATR) and carboxy-atractyloside [30,54] (Fig. 2). Carboxy-atractyloside is found only in fresh tubers and thermally decomposes to ATR [55]. ATR is a diterpenoid glycoside that occurs naturally in several plants found in Africa, Europe, Asia and South America [32]. ATR was first isolated in the Mediterranean thistle (*Atractylis gummifera*) [56], and there have been several cases of human poisonings in Europe due to the ingestion of this plant [57,58]. Poisoning with *A. gummifera* produces the same clinical picture as *C. laureola*, resulting in hepatic and renal necrosis. The accidental ingestion of another ATR-containing plant, *Xanthium strumarium* (cocklebur), has been shown to cause centrilobular hepatic degeneration and necrosis in grazing farm animals [59–61].

The biochemistry and toxicity of ATR has recently been reviewed [32,62]. ATR specifically binds to the adenine nucleotide translocator in the inner mitochondrial membrane and competitively inhibits the transport of ADP and ATP, thereby blocking oxidative phosphorylation [63]. The complete mechanism of toxicity of ATR, however, is not fully understood. Recent evidence suggests that ATR triggers apoptosis by inducing the mitochondrial membrane permeability transition pore, and the release of cytochrome c and caspase-activating proteases [64–68]. Numerous investigations have demonstrated that pure ATR is a potent hypoglycemic and nephrotoxic agent in experimental animals [32,69]. To date, however, there is no evidence to support that pure ATR causes the centrilobular liver necrosis that is characteristically seen in clinical cases of *A. gummifera* and *C. laureola* poisonings. While ATR-containing plants have been shown to produce hepatotoxicity in *in vivo* and *in vitro* studies [58,70], data on the hepatotoxic effects of pure ATR are limited.

Few experimental studies have investigated the *in vivo* toxicity of *C. laureola.* Wainwright *et al.* demonstrated that aqueous and methanol extracts of *C. laureola* produced centrilobular liver necrosis and early renal tubular necrosis in rats when given by intraperitoneal or subcutaneous injections [26]. The toxic components of the tuber are thought to be very polar in character since hexane and ether extracts have been shown to be nontoxic [26,48]. The investigators isolated a purified form of ATR from the methanol extract, and while this caused renal tubular necrosis, it failed to produce any hepatotoxicity [26]. Bhoola also found no liver changes in rats given ATR through intraperitoneal injections [42]. It has therefore been suggested that *C. laureola* contains at least two toxins: a nephrotoxin and a hepatotoxin, the latter which, to date, remains unidentified [26,71]. It is also possible that other active constituents of the plant and/or their metabolites modulate the effects of ATR *in vivo* [32]. Brookes *et al.* [49] identified the 6′-isovalerate esters of both ATR and carboxyatractyloside in the rootstock of *C. laureola.* Subsequent work by the same group led to the isolation of two new thymol derivatives and a ketol degradation product in the root as well [50]. The role that these constituents may have in the hepatotoxicity of *C. laureola* has not yet been determined. Data on the pharmacokinetics of ATR and *C. laureola* are virtually absent and whether other toxic metabolites are involved awaits further investigation.

Cases of human poisoning with *C. laureola* have been researched by various investigators in South Africa and are well documented in the literature [17,23,25,39]. Diagnostic methods to confirm such poisonings are in the process of development [23]. The experimental work conducted in the 1970s by Wainwright [26] is the only study to date demonstrating the toxic effects of *C. laureola* in rats *in vivo.* Despite clinical observations of the significant toxicity caused by this commonly used herb, no subsequent animal or *in vitro* investigations have been carried out; and the mechanism by which *C. laureola* produces hepatotoxicity is still not known. Therefore, as a starting point, we have made the first attempt to investigate the hepatotoxic effects of *C. laureola in vitro.* Although the *in vivo* rat model may often

serve as a valuable experimental tool, in the present study we chose to investigate the toxic effects of *C. laureola* in the human hepatoblastoma Hep G2 cell line. Previous work in our laboratory has established the Hep G2 cell line as an excellent model to study drug-induced hepatotoxicity [72–74]. This cell line offers an ideal system to examine the toxicity of *C. laureola;* first, because it is human-derived and thus avoids interspecies differences; and second, because the cell line is specific to the liver, the primary target organ of *C. laureola*-induced toxicity. Furthermore, Hep G2 cells in culture have been shown to retain the morphologic and most of the functional features of normal human hepatocytes [75]. In the present study, we report preliminary results demonstrating the hepatotoxic effects of *C. laureola* in human hepatocytes *in vitro.*

2. Methods

2.1. Materials

Dried powder from the rootstock of *Callilepis laureola* was kindly provided by Dr. M.J. Stewart (Toxicology Unit, Department of Chemical Pathology, South African Institute for Medical Research, University of Witwatersrand, Johannesburg, South Africa). MTT (formazan 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was obtained from Sigma Co. (St. Louis, NO, 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. PBS (phosphate buffered saline without Ca^{2+} or Mg^{2+}) 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 Co.

2.2. Preparation of plant extract

We opted to prepare an aqueous extract of *C. laureola* using a method that most closely resembles that of traditional Zulu healers [23,30]. An aqueous extract was prepared at a stock concentration of 40 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. Preliminary experiments demonstrated no cytotoxic effect of distilled water on Hep G2 cells at the concentrations used in the dilutions of *C. laureola.* Fresh plant extracts were prepared before each experiment.

2.3. Hep G2 cell line

Hep G2 cells were obtained from Wistar Institute (Philadelphia, PA, USA). Cells were seeded in collagen-coated Falcon flasks (1×10^6 cells/mL) [74]. 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% O_2 –5% CO_2 at 37°C. The pH of the media was maintained at 7.4. At 70 to 80% confluence, cells were trypsinzed 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 the *C. laureola* extract.

2.4. Experimental design

Control cells were exposed for 24 h only to plain essential medium (α-MEM), while the treated cells were incubated with different concentrations of the *C. laureola* extract (0.3, 0.8, 1.7, 2.5, 3.3, 5.0, 6.7 and 13.3 mg/mL). In a second set of experiments, cells were incubated for a total of 48 h. All components were filter-sterilized, and the entire procedure was conducted under aseptic conditions. To assess the time course of *C. laureola*-induced toxicity, cells were incubated for 1, 3, 6, 12, 18 and 24 h with either the LC_{50} concentration of *C. laureola* (the concentration that produced 50% cytotoxicity in Hep G2 cells) or the LC_{100} concentration of *C. laureola* (the concentration that caused 100% cytotoxicity in Hep G2 cells).

2.5. Cytotoxicity assay

Cytotoxicity was assayed using the MTT test. The methods of Mosmann [76] and Carmichael [77] were used with our modifications for cells grown directly in the 96-well plate. MTT (100 μL 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 μL 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%. For the treated cells viability was expressed as a percentage of control cells. All determinations were carried out in sextuplet in each plate. Due to a limited quantity of *C. laureola,* two plates were deemed sufficient for each experiment.

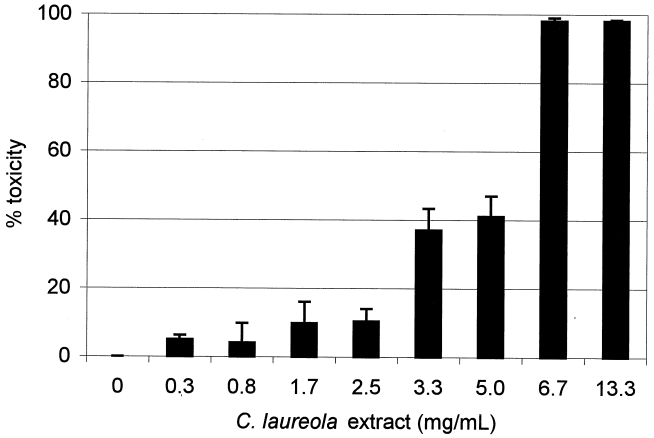

Fig. 3. Dose-dependent toxicity induced by *C. laureola* in Hep G2 cells. Hepatocytes plated directly in 96-well trays were exposed for 24 h only to plain medium (control) or to an aqueous extract of *C. laureola*. Cytotoxicity was assessed by the MTT test and expressed as a percentage of the control. Each bar represents the mean ± SD of 12 wells (6 wells/plate × 2 plates). Cytotoxicity was significant at all concentrations tested ($p < 0.05$ versus control at 0.3–2.5 mg/mL and $p < 0.0001$ versus control at 5.0 − 13.3 mg/mL).

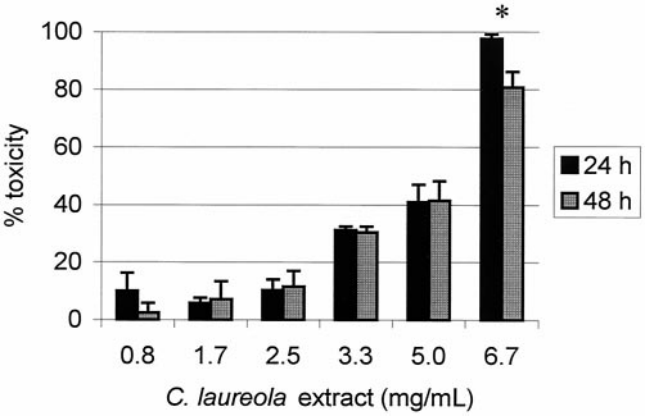

Fig. 4. Cytotoxic effects of prolonged exposure to *C. laureola* in Hep G2 cells. Hepatocytes plated directly in 96-well trays were exposed for either 24 h or 48 h to an aqueous extract of *C. laureola*. Control cells were exposed only to plain medium. Cytotoxicity was assessed by the MTT test and expressed as a percentage of the control. Each bar represents the mean ±SD of 12 wells (6 wells/plate × 2 plates). At the highest concentration tested (6.7 mg/mL), toxicity was significantly reduced after 48 h of continuous exposure to *C. laureola* compared to 24 h ($^{*}p < 0.01$). No differences in toxicity were observed at all other concentrations tested.

2.6. Statistical analysis

All data are expressed as means ± standard deviation (SD). Differences between control and treated cells and between 24 and 48 h incubation periods were analyzed using the Student's *t*-test. A p value < 0.05 was considered significant.

3. Results

A 24 h incubation with the *C. laureola* extract produced significant toxicity in Hep G2 cells at all concentrations tested ($p < 0.05$ vs. control at 0.3–2.5 mg/mL and $p < 0.0000001$ vs. control at 3.3–13.3 mg/mL, Fig. 3). Cytotoxicity was dose dependent with an LC_{50} of 3.5 mg/mL. Nearly 100% toxicity was observed at a concentration of 6.7 mg/mL. In a subsequent set of experiments, cells were also incubated for a prolonged period (48 h) with *C. laureola* (0.8−6.7 mg/mL, Fig. 4). After a 48 h incubation, the highest concentration of *C. laureola* (6.7 mg/mL) produced significantly less toxicity compared to a 24 h incubation (24 h incubation: 98% toxicity at 6.7 mg/mL; 48 h incubation: 81% toxicity at 6.7 mg/mL, $p < 0.01$). No differences in toxicity were observed between 24 and 48 h incubation periods at all other concentrations tested. The toxic effect of *C. laureola* in Hep G2 cells was found to be time-dependent (Fig. 5). Treatment with the LC_{100} concentration of *C. laureola* (6.7 mg/mL) produced 100% toxicity within 6 h.

4. Discussion

Medicinal plants undoubtedly have a valuable role in the treatment of disease, especially in under-developed countries. As with most therapeutic drugs, however, there is also a potential to cause toxicity. The toxicity of *C. laureola* has been documented in numerous clinical case reports, however the mechanism of toxicity is not known and until now there have been no published data available on the hepatotoxic effects of *C. laureola in vitro.*

We have previously reported that the Hep G2 cell line is a reliable *in vitro* model for the study of drug and alcohol-induced hepatotoxicity [74,75,78]. In the present work, we used this cell line to investigate the cytotoxic effects of *C. laureola in vitro.* An aqueous extract of *C. laureola* produced direct toxicity in a dose- and time-dependent manner, causing 100% toxicity within 6 h. These findings may explain the critical role that dosage appears to play in cases of *Impila* poisoning [25,53], in addition to the acute liver damage (within 24 h) that has been observed in experimental rats [26] and human poisoning cases [23].

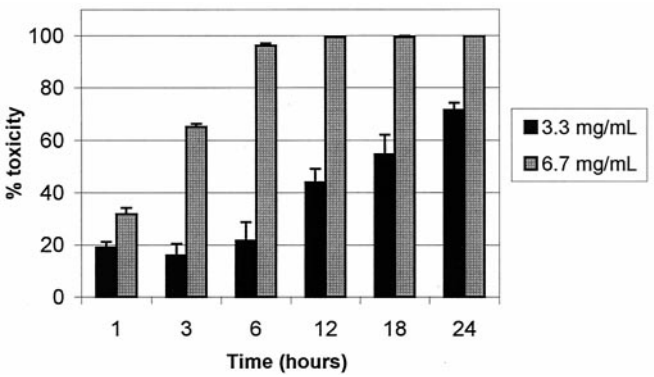

Fig. 5. Time course of the cytotoxic effect of *C. laureola*. Cytotoxicity of Hep G2 cells exposed to either 3.3 mg/mL or 6.7 mg/mL of *C. laureola* for 1 to 24 hr was assessed by the MTT test and expressed as a percentage of the control. Each bar represents the mean ±SD of 4 wells.

At concentrations ranging from 0.3 to 5.0 mg/mL, no difference in *C. laureola*-induced cytotoxicity was found between 24 and 48 h incubation periods. At the highest concentration (6.7 mg/mL), however, we actually observed a decrease in toxicity after 48 h of treatment compared to 24 h. It is possible that some of the toxic constituents of *C. laureola* are eliminated within a 24 h period. Additionally, the greater extent of damage produced by higher concentrations of *C. laureola* may induce hepatocyte regeneration. New hepatocytes that are formed over a 48 h period may have greater detoxification abilities, thus accounting for the reduced toxicity observed after prolonged exposure to *C. laureola.*

The MTT assay essentially measures the activity of the mitochondrial enzyme, succinate dehydrogenase, and is indicative of mitochondrial viability [76]. Our results therefore suggest that the principle target of *C. laureola*-induced toxicity is the mitochondria. This is an expected finding since ATR, a well known inhibitor of oxidative phosphorylation, is found in the tuber of *C. laureola* [71]. Several other constituents of the rootstock of *C. laureola* have been identified through thin layer chromatography [26,48,50]. These constituents, however, have not yet been tested for toxicity, and their potential involvement in hepatocyte damage is not known.

The mechanism by which *C. laureola* causes centrilobular liver necrosis is another area requiring further investigation. Evidence suggests atractyloside induces opening of the mitochondrial permeability transition pore, cytochrome c release and caspase activation [64–68]. Therefore, it is possible that *C. laureola* exerts its toxic effects through a similar apoptotic mechanism, perhaps involving the formation of reactive metabolites. Whether apoptosis or necrosis is the predominant mode of hepatocyte death involved in *C. laureola* intoxication will have clinically important implications for treatment interventions and the development of antidotes. The *in vitro* model used in the present study will be a useful tool to study the mechanism of *C. laureola*-induced hepatocyte death, and further investigations in this direction are currently in progress.

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