An active ingredient of Cat's Claw water extracts Identification and efficacy of quinic acid

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Abstract

Historic medicinal practice has defined Cat's Claw, also known as Una de Gato or *Uncaria tomentosa*, as an effective treatment for several health disorders including chronic inflammation, gastrointestinal dysfunction such as ulcers, tumors and infections. The efficacy of Cat's Claw was originally believed, as early as the 1960s, to be due to the presence of oxindole alkaloids. However, more recently water-soluble Cat's Claw extracts were shown not to contain significant amounts of alkaloids (<0.05%), and yet still were shown to be very efficacious. Here we characterize the active ingredients of a water-soluble Cat's Claw extract called C-Med-100 as inhibiting cell growth without cell death thus providing enhanced opportunities for DNA repair, and the consequences thereof, such as immune stimulation, anti-inflammation and cancer prevention. The active ingredients were chemically defined as quinic acid esters and could also be shown to be bioactive in vivo as quinic acid.
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1. Introduction

Uncaria tomentosa commonly known as Una de Gato or Cat's Claw has been widely used historically as a natural remedy, and it is currently present in a number of nutritional formulations to treat a large variety of health disorders (Blumenthal, 2003). So far to our knowledge most of the commercial preparations of Cat's Claw were based on oxindole alkaloid content (Keplinger et al., 1999), and not on their water solubility, bioavailability, clinical efficacy or lack of alkaloid content such has been the case with Cat's Claw water extracts such as C-Med-100® or Activar AC-11® (Sheng et al., 2000B; Sandoval et al., 2002).

The precise chemical identification of the active ingredients of C-MED-100® and Activar AC-11® have not been achieved as yet, but their chemical and biological characteri-

zation has been completed enough to standardize their commercial manufacture (Pero, 2000). They were formulated to mimic historical medicinal use of which the most important step is exhaustive hot water extraction for 18 h at 95 °C, and were spray dried to contain 8–10% or 16–20% carboxy alkyl esters (CAEs), respectively, as the only active ingredients found to be present. Daily oral doses of C-Med-100® in humans between 250 and 700 mg have been shown consistently to be efficacious (Lamm et al., 2001; Sheng et al., 2000A, 2000B, 2001).

The CAEs in C-Med-100 gave profound nutritional support as a dietary supplement because they enhance both DNA repair and immune cell responses, which in turn are critical physiological processes that regulate aging (Sheng et al., 2000B and as cited above). Both of these processes involved regulating the nuclear transcription factor kappa beta (NF-$\kappa\beta$). NF-$\kappa\beta$ is well known to control both the nuclear events that salvage cells from apoptotic cell death as well as from pro-inflammatory cytokine production (Beg and Baltimore, 1996; Wang et al., 1996). Hence, this mechanism directly

connects induction of apoptosis to programmed cell toxicity with inhibition of pro-inflammatory cytokine production and inflammation.

Apoptosis is another essential biochemical process in the body that regulates cells from division (replication) into differentiation and toward an increased functional capacity. Cells entering apoptosis will not only be stimulated to differentiate and take on function but they will eventually die from this 'programmed cell death'. Thus, increased apoptosis resulting from NF-κβ inhibition by C-Med-100® would both (i) effectively kill tumor cells because they would be forced out of replication by apoptosis and into eventual death, and (ii) at the same time increase immune cell responsiveness because more immune competent cells would be forced to differentiate and live longer by the paralleled enhancement of DNA repair. NF-κβ also sends signals to inflammatory cells to initiate them to produce cytokines (e.g. TNF alpha and the interleukins) that in turn stimulate phagocytic cells to kill more invading infectious agents, which at least in part is accomplished by producing pro-oxidant-generating inflammatory cytokines. Thus inhibiting NF-κβ has anti-inflammatory properties because it prevents over-reaction of the inflammatory process that can be very harmful to normal tissues of the body.

In addition, because pro-inflammatory cytokines are a major source of endogenous free radical production in humans, NF-κβ inhibition is expected to be anti-mutagenic by reducing genetic damage that can accumulate over time. The consequences would be that aging is curtailed because fewer radicals are produced to damage the DNA and to inhibit its natural repair. As a result, C-Med-100® should be considered an ultimate nutritional supplement for anti-aging remedies because it prevented free radical damage via NF-κβ inhibition, induced differentiation and immune cell responsiveness via the apoptotic pathway, enhanced DNA repair, and killed tumor cells, all of which in turn are major factors of why we age (Sandoval-Chacon et al., 1998; Sheng et al., 1998; Sandoval et al., 2000; Åkesson et al., 2003B). Here it is further disclosed that the CAEs characterized as the active ingredients of C-Med-100 have been identified and structurally elucidated as quinic acid analogs.

2. Materials and methods

2.1. Source of extracts of Uncaria tomentosa (Cat's Claw)

The primary historic medicinal preparation of Uncaria tomentosa involves heating bark to near boiling temperatures covered in water overnight, decanting the partially evaporated water extract and drinking it as a tea. Whereas such ethanopharmacological preparations have been shown repeatedly to be efficacious, high concentrations of tannins have contributed significantly to their toxic side effects by oral administration. However, this problem has been circumvented in water extracts of Uncaria tomentosa (Cat's Claw) destined for human consumption by utilizing C-Med-100® and Activar AC-11® supplied by Optigenex, Inc. (New York, NY) for these studies. C-Med-100® and Activar AC-11® were manufactured by a proprietary ultrafiltration process (Pero, 2000) that contained 8–10% CAEs or 16–20% CAEs, respectively, having no components >10,000 MW and that were essentially free of indole alkaloids (0.05%) (Sheng et al., 2000B; Sandoval et al., 2002, and this study). In order to chemically stabilize the manufacturing process the water extracts were spray dried on maltodextrin and finally processed into tablets or capsules. Whereas these extracts were 100% water soluble and thus directly bioavailable, they were also mimetic of ethnopaharmacological preparations only depleted of potential toxic side effect components.

2.2. Chemicals

Quinic acid (QA) was from Sigma and quinic acid lactone (QAL) was synthesized to 99% purity by Professer Robert Kane at Baylor University in Waco, Texas. Ammonia-treated QA (QAA) was made by neutralizing QA with 1% ammonia and then lypholyzing to dryness.

2.3. Isolation and purification of the active ingredient of water-soluble extracts of Cat's Claw, e.g. C-Med-100®

There are two commercial formulations of water-soluble Uncaria tomentosa extracts called C-Med-100® and Activar AC-11® standardized to 8–10% CAEs and 16–20% CAEs, respectively. Briefly, they are produced from heating 150 g of bark in 5 l of tap water for 12 h at 95 °C, decanting the soluble fraction, ultrafiltrating the resulting water extract to remove all components >10,000 MW and larger, and finally drying the <10,000 MW fraction according to U.S. Patent 6,039,949. For chemical analysis of the active ingredients in water extracts of Cat's Claw, they were first dissolved in water and then the spray drying agent (maltodextrin) removed by precipitation with 90% aqueous ethanol. The resultant solution was spotted on thin layer chromatographic (TLC) silica gel 60 F_{254} plates, and then chromatographed in a system of 1% ammonia in 95.5% ethanol. The TLC plate was scraped in 1-cm sections from baseline to solvent front, followed by elution of each section with 1% aqueous ammonia. Elution from silica gel 60 F_{254} TLC plates with aqueous ammonia proved to be necessary because of very tight binding of the active ingredient to silica. Although the $R_f = 0.3$ spot was essentially free from other Cat's Claw components, it contained relative large amounts of dissolved inorganic silica. In order to remove the inorganic component(s) introduced from the purification scheme off from silica TLC plates, the 1% aqueous ammonia solution was freeze dried and then the eluant redissolved in methanol leaving behind the solubilized silica. Distilled water was added to the methanol eluant, then again freeze-dried, before redissolving in water again for bioassay of tumor cell growth inhibition using HL-60 cells as already

described (See Section 2, Sheng et al., 1998). The only biological activity identified was located at $R_f = 0.3$ in the TLC chromatograms. The $R_f = 0.3$ compound had an ultraviolet absorption maximum at about 200 nm, and was absorbed onto charcoal, crystallized from methanol at −20 °C and further characterized chemically as a CAE by reaction with hydroxylamine and ferric chloride (See Section 2, Bartos reaction).

2.4. Analytical chemical analysis of the active ingredients in Cat's Claw water extracts

The crystalized active ingredient (about 1 mg) was completely dissolved in 0.7 ml D$_2$O for NMR with no shift reagent added. The following NMR spectra were recorded: 1: ^{1}H, 2: ^{1}H/^{1}H-correlated spectra (COSY), 3: ^{1}H/^{13}C-correlated spectra (HMBC), 4: ^{13}C-Dept 135 and 5: ^{1}H/^{13}C-correlated spectra (HMQ). A mass spectrum (MS) analysis was also performed by introducing the sample into the MS by infusion.

2.5. CAE determination by UV absorbance maxima at 200 nm in water-soluble extracts of Cat's Claw

The principle of this estimate of CAE was based on the fact that C-Med-100 has an absorption maximum of 200 nm. Because all the biological activity associated with C-Med-100® was confined to compounds absorbing at 200 nm, and they were positive for an ester linkage, then the CAE content could be estimated by quantifying the 200 nm absorption against a standard simple benzoic acid-type CAE, which in this case was dioctyl phthalate. According to this method, dissolve 100 mg C-Med-100 in 1 ml of distilled water for 30 min. Centrifuge at $2000 \times g$ for10 min and save the supernatant for analysis. Take 200 μl from the C-Med-100® supernatant and add 4.8 ml of 99.7% ethanol. The resulting solution contained 4 mg/ml C-Med-100® suspended in about 96% ethanol. Vortex (mix) and centrifuge at $2000 \times g$ to remove insoluble material. The supernatant was diluted from 4 to 30–200 μg/ml with 99.7% ethanol for measurement of UV absorbance at 200 nm. Depending on the particular CAE content in C-Med-100®, you should normally examine 200 nm absorption of 60–120 μg/ml in duplicate for calculation of CAE against a dioctyl phthalate standard in the range of 0–50 μg/ml.

2.6. (Quinic acid) QA ester determination in water-soluble extracts of Cat's Claw by Bartos reaction

There were three reagents necessary to quantify the level of QA esters in C-Med-100® according to the procedure presented by Bartos (1980). The chemical principle behind this procedure was first to convert the QA esters to hydroxamic acids with hydroxylamine reagent (5 ml of a 10% hydroxylamine hydrochloride in methanol + 10% sodium hydroxide in methanol (pH 10), diluted to 15 ml in methanol and finally filtered through Munktell 00M, prepared fresh). Next, the resulting hydroxamic acids were reacted with a ferric chloride reagent (0.3% ferric chloride hexahydrate in a 3% v/v solution of 70% perchloric acid in ethanol) to produce a chromophore absorbing at 490 nm. The color intensity was read at 490 nm against a standard of QAL (10 mg/ml and add 0, 12.5, 25, 50, and 75 μl up to 1000 μl in 95% ethanol). Further details were to add 1 ml of C-Med-100® sample or QAL standard dissolved in ethanol to 0.5 ml of hydroxylamine reagent and let stand in dark at room temperature for 30 min. After this incubation another 3 ml of ferric chloride reagent was added, again placed in the dark for an additional 15 min, and the absorbance at 590 nm read in a spectrophotometer against the various concentrations of QAL standard.

2.7. NaOH neutralization analysis of QA equivalents in water-soluble extracts of Cat's Claw

Another possible way to estimate how much QA exists in a free acid form in C-Med-100® was to neutralize with NaOH to determine how many equivalents of base it took to adjust the pH of C-Med-100® to pH 7. For this purpose, the spray drying agent, maltodextrin, was first removed from C-Med-100® extract because it contributed artificially to the acidity of C-Med-100®. This was accomplished by dissolving 5 g C-Med-100® in 50 ml of distilled water for 30 min, and then to precipitate out the maltodextrin with 950 ml of 99.7% ethanol. After filtration through Munktell 3 filters and evaporation of ethanol, the resulting solution was freeze dried and used directly to estimate QA equivalents as free acid by neutralization with 1N NaOH. Only carboxy acids having an UV absorption maximum at 200 nm such as QA or QA esters (with free carboxyl groups), were potentially measured, because charcoal adsorption removed the $A_{200\,nm}$ material (Fig. 1), and there was also no QA present nor any biological activity remaining either. Milliquivalents of NaOH-neutralized de-maltodextrinized C-Med-100® were calculated, and then converted to the percentage acid present in the original C-Med-100® based on a w/w basis.
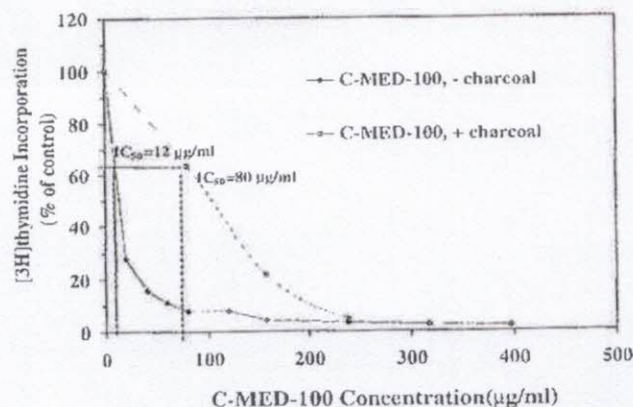

Fig. 1. The influence of UV-absorbing compounds removed by charcoal adsorption of the HL-60 cells in vitro. The HL-60 bioassay is presented in detail elsewhere (Sheng et al., 1998).

2.8. In vitro evaluation of the efficacy of active ingredients found in water-soluble Cat's Claw extracts

Based on the chemical evidence so far collected, we have undertaken to demonstrate by in vitro bioassays direct biological proof that QA analogs were indeed responsible for the efficacy observed with C-Med-100®. It has already been established both in vitro and in vivo that cells or animals treated with C-Med-100® accumulate cells both in peripheral circulation and the spleen, and this accumulation has been explained by cell proliferation being inhibited, but without inducing cell death (Sheng et al., 2000A; Åkesson et al., 2003A, 2003B). This mechanism of action has been evaluated in this study by comparing cells dying from damage during replication to cells not growing, but yet still accumulating because the cells that normally die off during replication as they mature and age are still alive and apparently healthy, but yet not growing (Åkesson et al., 2003A, 2003B). Here, we evaluate cell growth in human mononuclear leucocytes (HML) to estimate cell survival in an essentially G_0 nondividing cell population, and in an exponential growing cell population of human leukemic HL-60 cells having a high S-phase (replication) population of cells using the MTT vital staining colorimetric bioassay. MTT is taken up by viable cells and does not distinguish between cells that are replicating and those that are not replicating, but yet only dying cells are not stained. Hence, MTT staining of HML after treatment with bioactive ingredients of C-Med-100® was used to score increased survival by estimating the total MTT-positive non-replicating cells. The response of MTT-positive HML was quantified by reporting the dose of bioactive ingredients it took to increase the vital staining colorimetric value by a factor of 2, (i.e. = double # of cells). On the other hand, exponential growing HL-60 cells exposed to bioactive ingredients of C-Med-100 and then MTT stained could measure growth inhibition of a dividing cell population if the control (untreated) MTT-stained HL-60 cells was greater than after treatment with bioactive ingredients. In such a case, the MTT-stained untreated HL-60 cells were compared to those exposed to serial doses of active ingredients, the dose–response curves plotted and the data recorded as IC_{50} values. The isolation and culturing of HML from peripheral blood samples (Pero et al., 1996), and the culturing, maintenance of exponential growth of HL-60 cells as well as further experimental details of MTT bioassay procedure have been presented elsewhere (Sheng et al., 1998).

2.9. In vivo evaluation of the efficacy of QA as an active ingredient isolated from water-soluble Cat's Claw extracts

A rat model was used to evaluate if active ingredients isolated from water-soluble extracts of Cat's Claw could be shown to be effective in vivo by inducing recovery of peripheral white blood cells (WBC) after doxorubicin DXR)-induced leucopenia. This model has already been used ef-

fectively to demonstrate C-Med-100® administration after DXR treatment enhanced recovery of WBC about as efficiently as Neupogen, a standard therapy used in the clinic for this purpose (Sheng et al., 2000). The rat study experimental design used to compare QA and C-Med-100® is outlined in diagrammatic form below.

2.9.1. Aim
To assess the effect of quinic acid (QA) and ammonia-treated quinic acid ammonium (QAA) compared to C-Med-100® on chemotherapy (Doxorubicin)-induced leucopenia.

2.9.2. Animals
Fifty female W/Fu rats, weighing 150–180 g.

2.9.3. Drug and administration:
1. Doxorubicin: from Pharmacia and Upjohn, given 2 mg/kg for groups 2–5, i.p. injection (days 1, 3, 5).
2. C-Med-100® (batch no. E-40622), 80 mg/kg, gavage daily from day 6 (24 h after the third/last treatment of DXR) to the end of experiment (day 21/22).
3. Quinic acid (QA, Sigma): 200 mg/kg, gavage as C-Med-100®.
4. Ammonia-treated quinic acid (QAA) synthesized as described in Section 2 and administered at 200 mg/kg, gavage as C-Med-100®.

2.9.4. Endpoint measurements
1. Body weight (GM): before and at the end of the experiment.
2. Blood sampling: day 0 (before any treatment), day 4 (24 h after the second DXR treatment), day 7 (48 h after the third/last DXR treatment), day 11, and day 15. Whole peripheral blood samples were collected into K_3-EDTA containing tubes by periorbital puncture and then analyzed for WBC within 1 h with an automated hematology analyzer (Sysmex, K-1000). Organ weights from major tissues (liver, kidney, lung, heart, spleen) were also collected and used as an indicator of any toxicity.

Group	N	Doxorubicin (i.p.)	Supplement drug (gavage)
1. Control	10	Saline control	Sterile tap water
2. Doxorubicin (DXR)	10	2 mg/kg × 3	Sterile tap water
3. C-Med-100® (80 mg/kg)	10	2 mg/kg × 3	C-Med-100
4. DXR + QA (200 mg/kg)	10	2 mg/kg × 3	QA (200 mg/kg)
5. DXR + QAA (200 mg/kg)	10	2 mg/kg × 3	QA-ammonium (200 mg/kg)

3. Results

3.1. Identification of quinic acid (QA) as an active ingredient of water extracts of Cat's Claw, e.g. C-Med-100®

The characterization, isolation and final purification of the bioactive component in Cat's claw water extracts by silica gel TLC has already been presented in Section 2. Here, our laboratory presents the additional chemical and biological evidence that the only bioactive ingredient found was QA. Firstly, it was important to establish that the only area on silica gel 60 F_{254} TLC plates chromatographed in 1% ammonia in 95% ethanol that had biological activity assessed by the HL-60 bioassay was at $R_f = 0.3$. Because the 1 cm scraped TLC plate sections were eluted from the silica gel with 1% ammonia, any acids or esters present at this R_f location would have been converted to an ammonium salt, and this analog would have had the biological activity attributed to C-Med-100®. Nevertheless, the ammonia eluant was freeze dried, re-dissolved in water and the UV-spectrum determined to have an absorption maximum at 200 nm.

Because of the possibility of base hydrolysis of esters or ammonium salt/chelate formation of acids of the bioactive component, our lab next wanted to determine the UV absorption maximum of C-Med-100® dissolved in water but not treated with ammonia. C-Med-100 also had an UV absorption maximum of 200 nm in water or ethanol. These data led our laboratory to determine if we removed the UV-absorbing material from C-Med-100® we would then also remove the biological activity. For this purpose, a comparison of C-Med-100® water solutions before and after activated charcoal absorption (1 gm/1 ml C-Med-100®/gm charcoal) was carried out. The data from such an experiment are presented in Fig. 1. Here, it can be seen that >85% of the in vitro HL-60 cytotoxicity in C-Med-100® extract was removed due to charcoal absorption, and likewise it was paralleled by removal of >85% 200 nm UV-absorbing materials. Since the only 200 nm UV-absorbing components in C-Med-100 were located at $R_f = 0.3$, and since C-Med-100® had a 200 nm UV absorption maximum which if removed by charcoal absorption, also destroyed its biological activity, it was concluded that the active ingredient of C-Med-100 could only be attributed to components absorbing at 200 nm and that travelled on silica gel 60 F_{254} TLC plates chromatographed in 1% ammonia in 95% ethanol to $R_f = 0.3$.

The C-Med-100® bioactive component at $R_f = 0.3$ was crystallized from methanol and subjected to analytical chemical analysis. NMR analyses indicated the ^{1}H-spectrum contained signals from a main compound. The three ^{1}H-signals at 1.72–1.99, 4.03, 3.90 and 3.43 ppm and Cosy spectra were found to be signals from methine-groups and ^{13}C-signals at 66.9–75.1 correlated to protons found in CH_2- and CHOH-groups in a straight-chain confirmation and correlated to ^{13}C-signals at about 40 ppm. The three ^{1}H-signals are also bound to each other in a straight chain as found in the COSY spectrum. MS analyses were also performed the D_2O solution diluted with acetonitrile (ACN) (50/50), which gave the mass number of 197 (negative ions, M–D = 195). After evaporation the mass number 192 was achieved (negative ions, M–H = 191). An authentic standard comparison of the NMR and MS analyses confirmed QA to be the active ingredient of C-Med-100® (Fig. 2).

3.2. Quantitative determination and in vitro biological evaluation of QA analogs in water extracts of Cat's claw; e.g. C-Med-100®

Because QA was determined as an active ingredient, combined with the uncertainty that QA might have arisen as a result of base hydrolysis elution from silica gel after TLC, a concerted effort was made to determine the presence of potential QA analogs in C-Med-100®. For this purpose, our lab has developed three chemical procedures that are capable of estimating various types of QA analogs that might be present in C-Med-100®, namely (i) CAEs by UV absorption at 200 nm quantified against dioctyl phthalate, (ii) QA esters using QAL as the standard ester and quantified by the Bartos reaction by forming hydroxamic acids and chromophores with ferric chloride, and (iii) by NaOH neutralization that in turn estimates any free acid equivalents present in C-Med-100®. The data of such a comprehensive chemical analysis are presented in Table 1, and they have indicated about 8–10% CAE esters (w/w) were present in C-Med-100®, of which 4–5% could be accounted for as QA esters. In addition, <1.6% (w/w) of C-Med-100® existed as the free QA analog (H+ form). It was concluded that either free QA existed as an active ingredient in C-Med-100® at <1.6%, or there was a QA ester analog which had a free non-esterified carboxyl group present. In either case, these data are consistent with the major active ingredients in C-Med-100 as being CAEs in the form of QA esters.
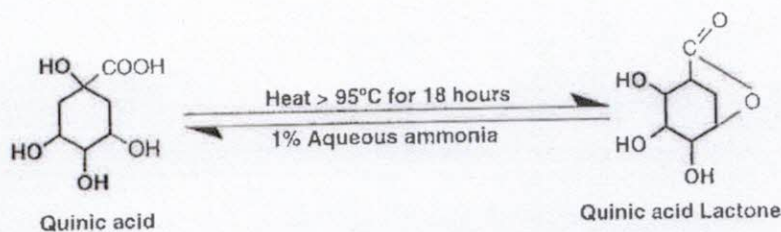

Fig. 2. The structure of quinic acid (QA) and quinic acid lactone (QAL) and their interconversion by heat at 95 °C and base hydrolysis by 1% aqueous ammonia.

Table 1
The average content of QA analogs in C-Med-100

C-Med-100 batch number	UV A_{200nm} method (dioctyl phthalate standard)) CAE est. (% w/w)	Bartos method (QAL ester standard) QA ester est. (% w/w)	pH method (NaOH neut.) free acid est. (% w/w)
E-42526	9.0	4.6	<1.6
E-43183	9.8	4.8	<1.7
E-43229	9.4	4.6	<1.8
E-43682	9.4	4.8	<1.4
E-43751	9.2	4.7	<1.9
E-44038	8.8	4.4	<1.6
E-44073	8.8	4.7	<1.9
Average	9.2	4.7	<1.7

In order to shed further light on the characterization of QA analogs as the active ingredients in C-Med-100®, our lab next determined the influence of base hydrolysis on the chemistry and biological activity of C-Med-100®. QAL is in fact a cyclic ester of QA, and as such it is an example representing the general class of QA esters. Elution from silica gel with 1% ammonia were the mandated chemical conditions necessary to remove the QA analog (i.e. active ingredient) from silica gel which in turn favored the base hydrolysis of QA esters such as QAL to QA. If so, then identification of QA after base hydrolysis from the elution of silica gel, would then be consistent with the natural occurrence in C-Med-100® of a QA ester at $R_f = 0.3$. Hence, it was undertaken to prove that QAL as an example of QA esters was indeed hydrolyzed by either 1N HCl or 1 M NaOH. The data are presented in Table 2. It is quite clear that either strong acid or base treatment converted QAL to QA, and left only QA remaining, thus supporting the fact that the isolation of QA from C-Med-100® after base elution from silica gel was likely to have been originally present as a QA ester.

Having confirmed that base hydrolysis converted QA acid esters to QA, our laboratory proceeded to quantify C-Med-

Table 2
The acid and base hydrolysis of QAL and QA

Compound	μg/test	Bartos reaction (A_{490} nm)			
		Initial	Average	−Blank	% Degraded
Blank	0	0.175	0.175	0	–
QA	500	0.170		±0	±0
QA	500	0.172	0.172		
QAL	500	0.742			
	500	0.752	0.750	0.575	–
QAL (1N HCl)	500	0.413			
QAL (1 M NaOH)	500	0.323	0.368	0.192	66.6
QA (1N HCl)	500	0.170	0.176		
QA (1 M NaOH)	500	0.175	0.169	±0	±0

The disappearance of the ester linkage in QAL was measured by formation of hydroxamic acids and colorimetric development with ferric chloride using the Bartos reaction. The lack of breakdown of QA was also confirmed by TLC and UV absorption at 200 nm.

100® for the presence of very types of QA analogs. The data in Table 1 utilize three separate chemical procedures to estimate the relative amounts of potential QA analogs found in unhydrolyzed C-Med-100®. First of all, there is maximally <1.6% QA in C-Med-100®, because by base neutralization there were only a total of <1.6% free acid equivalents, which also included all other acids that might have been present, thus contributing to the acidity of C-Med-100®. Hence, QA alone cannot account for the efficacy of C-Med-100®. However, there was a much more substantial amount of CAEs, and this class of compounds had already been shown to contribute to the efficacy of C-Med-100® (Fig. 1), and at least about 4–5% of the CAEs could be accounted for as QA esters (Table 1).

The final support that QA is not the natural-occurring bioactive QA analog in C-Med-100®, but rather it probably occurred as a QA acid ester as reported in Table 3. First we have observed that when QA was heated according to the manufacturing protocol for C-Med-100®, which was 8–12 h at 95 °C, QAL was formed suggesting the possibility that the active ingredient of C-Med-100® could in fact be the CAE identified as a QA ester such as QAL (Fig. 1). However, the in vitro efficacy data reported in Table 3 rule out QAL as well as QA as the primary active ingredients occurring naturally in Cat's Claw water extracts such as C-Med-100®; i.e. by comparison of the IC$_{50}$ values of 2300 μg/ml versus 500 μg/ml, respectively. However, if C-Med-100® was first hydrolyzed for 1 h with 1 M NaOH, neutralized with 1N HCl and then bioassayed, the biological activity was reduced in about the proportion that QA acid esters also disappeared from C-Med-100®, (Table 3). Furthermore, as the QA esters were broken down by the base hydrolysis, QA began to appear in the hydrolyzed extracts as judged by TLC analysis clearly indicating QA was present in ester linkage in C-Med-100®.

It has been postulated that the observed in vivo enhancement of DNA repair by C-Med-100® (Sheng et al., 2000B, 2001) could be due to allowing greater time to remove DNA lesions before cell death ensues from replication blocks or otherwise. The data in Table 3 are quite consistent with this mechanism. A prerequisite for enhancing DNA repair would be to slow down DNA replication but not induce any cell death to cells not in S-phase. When MTT analysis of cell populations were used to evaluate toxicity in -HML (non-dividing) or exponential growing HL-60 (high S-phase) cells treated with C-Med-100 HML doubled in number whereas HL-60 cells were growth inhibited. The IC$_{50}$ values were greatly increased after base hydrolysis strongly supporting that QA esters were responsible for this observed novel biological effect, and suggesting growth inhibition without cell death would be stimulatory to DNA repair.

3.3. In vivo efficacy studies of QA in the rat

There are two physiological factors regarding the natural forms of QA as the active ingredients of water extracts

Table 3
The coupling of the disappearance of in vitro biological efficacy of C-Med-100 assessed in HL-60 and HML cells to a corresponding disappearance in CAE content analyzed as QA esters by the Bartos reaction

Compound	HL-60 MTT IC_{50} (μg/ml)	HML MTT 2x #cells (μg/ml)	%QA est. (Bartos)	QA est. TLC identical
QA	2300	>2300	0	++++
QAL	2300	>2300	100	0
QAL + 2 M NaOH, 2 h	1900	>2300	35	++
C-Med-100 (no base hydrolysis)	536	500	4.7	±
C-Med-100 (M NaOH for 2 h)	900	1200	2.5	++

Table 4
Enhanced recovery of doxorubicin (DXR)-induced-leucopenia in the rat by treatment with C-Med-100 or quinic acid (QA)

Group[a]	N	Treatment and sampling schedule (10^{12} WBC /l)				
		Day 0	Day 4	Day 7	Day 11	Day 15
Control (saline, gavage)	10	9.5 ± 1.4	8.8 ± 0.9	9. ± 1.0	9.2 ± 1.1	9.0 ± 0.8[b]
DXR (2 mg/kg, i.p.)	10	9.5 ± 1.4	5.9 ± 0.7	5.3 ± 0.7	6.2 ± 0.7	7.1 ± 1.0
C-Med-100 (80 mg/kg, gavage)	10	9.6 ± 1.4	5.4 ± 0.7	5.7 ± 1.1	7.6 ± 1.7	8.4 ± 1.5[b]
QA (200 mg/kg, gavage)	10	9.5 ± 1.5	6.5 ± 1.1	5.5 ± 0.9	6.4 ± 0.7	8.1 ± 1.5[b]
QA-NH_3 (200 mg/kg, gavage	10	9.5 ± 1.1	6.5 ± 0.9	5.2 ± 0.5	6.7 ± 1.1	8.5 ± 1.0[b]

[a] DXR treatment days were 1, 3, and 5 followed by C-Med-100, QA or QA-NH_3 treatment for 6–11 days thereafter.
[b] $p < 0.05$ compared to DXR alone all other groups were not significantly different after 15 days of treatment.

of Cat's Claw such as C-Med-100® which in turn might result in quite different biological responses when administered in vitro or in vivo. Firstly, there was the pH 1 of the stomach that we have shown is strong enough to hydrolyze any QA esters present in C-Med-100® to QA (Tables 2 and 4). Secondly, the microflora of the digestive tract of mammals are well known to both synthesize and metabolically convert QA to other analogs such as chlorogenic acid, ferrulic acid, shikimic acid, cinnamonic acid, and benzoic acid (Seifter et al., 1971; Gonthier et al., 2003). These well-known physiologic facts have raised the possibility that even though QA esters are the bioactive ingredients in vitro, QA esters in vivo could have been metabolized to QA before being absorbed into circulation to mediate efficacious responses. In order to test the hypothesis that natural occurring QA esters identified in C-Med-100 might be catabolized to QA in vivo and yet still be bioactive, we have directly compared the efficacy of QA and QAA to C-Med-100® in a rat model. This in vivo model has already been used successfully by our laboratory to demonstrate that DXR-induced leucopenia is restored to normal WBC levels about as efficiently as standard therapy for this purpose (i.e., Neupogen®) after receiving oral daily doses of C-Med-100® of 40–80 mg/kg for 14 days (Sheng et al., 2000). Table 4 displays the data of rats given three doses of DXR (2 mg/kg) on days 1, 3, and 5 after initiation of the experiment, and then followed by oral daily administration of either water (control), C-Med-100®, QA or ammonia-treated QA (QAA) for 6–21 days thereafter. Peripheral blood WBC were recorded throughout this time period, and the data indicated both QA or QAA could induce recovery from DXR-induced leucopenia about as effectively as 80 mg/kg C-Med-100® could. Furthermore, weight loss and organ pathology did not indicate any toxicity for the doses of C-Med-100® or its active ingredients that were tested. Based on these data

it was concluded that a QA analog was at least one class of active ingredients also observed for water-soluble Cat's Claw water extracts (e.g. C-Med-100), albeit the exact in vivo QA bioactive form has not as yet been identified, although it can be substituted for by QA or QAA in vivo.

4. Discussion

Here we report on a new class of active ingredients found in Cat's Claw water extracts, namely QA analogs, and they were essentially devoid of any alkaloid content (<0.05%) (Sheng et al., 2000B; Sandoval et al., 2002), which in turn had been described earlier to be the bioactive components of Cat's Claw (Keplinger et al., 1999). It is likely that this apparent discrepancy is more related to differences in water solubility and bioavailability than anything else.

The in vitro and in vivo efficacy data reported on in this study (Tables 3 and 4) were not always mutually supportive. For example, the in vitro QA dose having activity had an IC_{50} value of 2300 μg/ml whereas the in vivo QA active dose that demonstrated efficacy was 200 mg/kg, which when extrapolated to an in vitro dose was about 200 μg/ml. Hence, although most of the in vivo efficacy of C-Med-100® could be accounted for by the in vivo efficacy of QA alone, a much larger discrepancy between efficacious in vitro doses of C-Med-100 and QA were shown to exist; i.e. being 500 and 2300 μg/ml, respectively. However, there is a very plausible explanation for these results that has also been put forward with the data recorded in Tables 1–3. Here it becomes obvious that the natural occurrence of QA in C-Med-100 is not as a free acid but in the form of QA esters because base or acid hydrolysis of C-Med-100 destroyed the biological activity and generated free QA. Because oral ingestion of C-

Med-100 could convert QA esters to QA at the pH 1 of the stomach, or be metabolized by non-specific esterases and the shikimate pathway existing intestinal microflora (Herrmann and Weaver, 1999), it follows then a primary in vivo active metabolic form of QA esters might in fact be QA via metabolic conversion of QA esters to QA in the GI track. Further experimentation is planned to resolve this complicated issue.

One highlight of this study was to present data that support the mode of action of C-Med-100® was also true for QA analogs. C-Med-100® and Activar AC-11® are water extracts of Cat's Claw registered with the Federal Trade Commission and reported in the literature to enhance DNA repair and immune function (Sheng et al., 2000A, 2000B; Lamm et al., 2001; Sheng et al., 2001; Åkesson et al., 2003A, 2003B). Åkesson and other laboratories have shown that water-soluble Cat's Claw extracts such as C-Med-100® inhibit NF-κβ (Sandoval-Chacon et al., 1998; Åkesson et al., 2003B) that in turn can lead to growth inhibition without cell death. These properties are growth-regulating conditions very favorable to DNA repair enhancement. Here in Table 3 it is reported that QA esters were active ingredients present in C-Med-100® that prolonged survival of G_0-cells and reduced replication of S-phase cells. Taken together, it becomes quite convincing that since QA analogs have similar modes of action and mediate quite the same biological responses leading to clinical efficacy, they are the major active ingredients of water-soluble Cat's Claw extracts.

Acknowledgements

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