Phenolic Compounds of *Chromolaena odorata* Protect Cultured Skin Cells from Oxidative Damage: Implication for Cutaneous Wound Healing

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Extracts from the leaves of *Chromolaena odorata* have been shown to be beneficial for treatment of wounds. The crude ethanol extract of the plant had been demonstrated to be a powerful antioxidant to protect fibroblasts and keratinocytes *in vitro*. In this study, the most active compounds were fractionated and identified from the crude extract using liquid chromatography coupled with UV spectroscopy and mass spectrometry. The antioxidant effects of purified fractions on cultured fibroblasts and keratinocytes were investigated using colorimetric and lactate hydrogenase release assay. The results showed that the phenolic acids present (protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids) and complex mixtures of lipophilic flavonoid aglycones (flavanones, flavonols, flavones and chalcones) were major and powerful antioxidants to protect cultured skin cells against oxidative damage. In conclusion, the extract from *C. odorata* contains a mixture of powerful antioxidant compounds that may be one of potential mechanism contributing to enhanced wound healing.

Key words *Chromolaena odorata* extract; oxidative damage; antioxidant; wound healing

Chromolaena odorata (L.) R. KING & H. ROBINSON (formerly *Eupatorium odoratum* L.), a perennial belonging to the plant family Asteraceae (=Compositae), is a diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures of southern Asia and western Africa. It forms a bush 3—7 m in height when growing in the open. Native to Mexico, the West Indies, and tropical South America, it was spread widely by early navigators. It is a weed of 13 crops in 23 countries.[1)] Traditionally, fresh leaves or a decoction of *C. odorata* have been used throughout Vietnam for many years as well as in other tropical countries for the treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis.[2)] A number of studies demonstrated that the extract of the leaves of *C. odorata* inhibited the growth of bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Neisseria gonorrhoea*).[2—6)] Enhancement of hemostasis and blood coagulation with use of *C. odorata* extract has also been reported.[3,7)] A clinical trial of *C. odorata* extract was conducted in the National Institute of Burns in Hanoi, Vietnam between 1987 and 1991 on 136 patients with full thickness wounds and an average wound size of 79.9 cm^2. The stimulatory effects of *C. odorata* extract on the formation of granulation tissue and wound re-epithelialization were demonstrated clinically and histologically.[2)] In previous *in vitro* studies we have shown that *C. odorata* extract inhibits the contraction of collagen lattices by normal human dermal fibroblasts—an *in vitro* model of wound contraction[8)] and enhances fibroblasts and endothelial cell proliferation.[9)] Human epidermal keratinocyte proliferation is stimulated by low concentrations (from 0.1 to 5 μg/ml) of the extract and cell differentiation at higher concentrations (from 50 to 300 μg/ml). In an *in vitro* model of re-epithelialization–scratch technique, keratinocyte migration is enhanced by the extract at intermediate concentrations (from 5 to 60 μg/ml).[12)] Using indirect immunofluorescence we demonstrated the up-regulation effects of the extract on human keratinocyte production of the basement membrane zone components and extracellular matrix proteins.[10)]

The antioxidant effects of the extract have previously been investigated. It was found that within the range of 50 to 800 μg/ml the extract showed significant protection on cultured fibroblasts and keratinocytes against hydrogen peroxide and superoxide radicals damage.[11)]

Several classes of flavonoids have been isolated from *C. odorata* extracts.[13—16)] Three flavanones and one flavone were isolated and proved to be responsible for blood coagulation.[7)] The extract also contains high concentrations of amino acids.[12)]

Oxidants are now considered to be involved in a number of aspects of burn injury and tissue repair. Oxygen free radicals contribute to further tissue damage in the events following skin injury and are known to impair healing process. Antioxidants, on the other hand, significantly prevent tissue damage and stimulate wound healing. The extract from *C. odorata* was scientifically demonstrated to have a strong anti-oxidant effect.[11)] In this study, we fractionated and identified the most active antioxidant components to get an insight into the mechanism of how the extract can be beneficial to wound healing.

MATERIALS AND METHODS

Media and Chemicals Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), streptomycin, penicillin, gentamycin, fungizone were from Gibco. Hydrogen peroxide, catalase, hypoxanthine, xanthine oxidase, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], sodium dodecyl sulfate (SDS), and *N*,*N*-dimethylformamide (DMF), phosphate buffered saline without Ca & Mg (PBS), epidermal growth factor (EGF), cholera toxin, hydrocortisone were purchased from Sigma Chemical Co. (U.S.A.). Dispase II was purchased from Boehringer Mannheim (U.S.A.). Keratinocyte Growth Medium was purchased from Clonetics (U.S.A.). The phenolic acid standards were purchased from Sigma, and the flavonoid standards from Apin, Abingdon,

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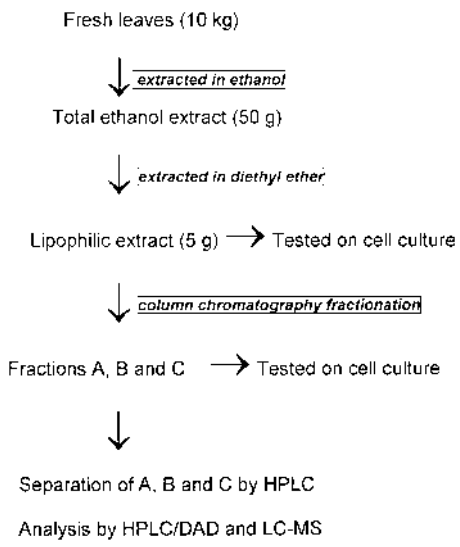

Fig. 1. Overview of the Fractionation and Identification of the Plant Extract

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Preparation of Crude Total Ethanol Extract Crude extract was prepared from fresh leaves of *C. odorata*. Method of extraction was described before.[11] Fresh leaves of *C. odorata* (10 kg) were collected, washed, dried in sunlight for 5 d then in 45 °C incubator for another 3 d. One kilogram of dried purified leaves were powdered, divided into 3 batches then extracted with 4000 ml of absolute ethanol in a Soxhlet apparatus for 12 h per batch. Twelve liters of the ethanol fraction was evaporated under vacuum using a Rotary Vacuum Evaporator, RE 100, Bibby, U.K. Chlorophyll, resin and lipids were removed by Whatman paper filtration and by extraction of the residue with petroleum ether in a Pyrex Separatory Funnel. One hundred grams of the dried residue were divided into 2 parts. One part (50 g) of the residue were shaken with five portions of diethyl ether to extract lipophilic substances. The diethyl ether was evaporated under vacuum to yield 5 g of a yellow thick extract. For cell culture experiments, this lipophilic extract was reconstituted in dimethylsulfoxide (DMSO; Sigma, U.S.A.), which was adjusted to a final volume in culture medium $<0.1\%$ (v/v). Various dilutions at 50, 100 and 200 μg/ml of the extract in the medium were prepared on the day of the experiment (Fig. 1).

Fractionation by Column Chromatography The extract obtained from the diethyl ether extraction after using a small amount of it for culture experiments was fractionated by column chromatography on Silica gel 60 (Merck), using different proportions of ethyl acetate/methanol as eluting solvents. Three out of four fractions were found to be strongly antioxidant when tested on the cell culture system (Fig. 1).

Separation of Constituents by Semi-preparative High Performance Liquid Chromatography (HPLC) The three antioxidant fractions obtained by column chromatographic fractionation were labeled A, B and C. Further separation of these fractions was performed by HPLC. Hewlett Packard HPLC equipment with Diode Array Detection was used (HP-1100). Separation was carried out using a C18 semi-preparative column at room temperature. A mixture of methanol and water was used as mobile phase with a flow-rate of 2 ml/min. Detection took place at the wavelengths of 254, 290 and 335 nm, and for the separations 100 μl of each fraction were injected. Different fractions were collected and lyophilized for chemical analysis by analytical HPLC coupled with a diode array detection (DAD) and atmospheric pressure chemical ionization mass spectrometry (APCI-MS).

Analysis of the Compounds by HPLC with DAD and APCI-MS The compounds obtained after separation of the constituents in A, B and C were analyzed by means of HPLC/DAD and HPLC/APCI-MS and the data obtained were compared with those of standards. The analytical HPLC system consisted of a Waters LC 600 pump and 996 photodiode array detector. A LiChrospher 100RP-18 (5 μm) column was used, 4.0 mm (i.d.)$\times$250. Gradient profiles based on two solvents, denoted A and B, were employed. A was 2% aqueous HOAc and B was MeOH–HOAc–H_2O, 18 : 1 : 1. Initial conditions were 75% A, 25% B, with a linear gradient reaching B$=$100% at $t=20$ min. This was followed by isocratic elution (B$=$100%) to $t=24$ min, after which the programme returned to the initial solvent composition. Column temperature was maintained at 30 °C, and a flow rate of 1.0 ml$\cdot$min^{-1} was used. All samples were injected by autosampler.

For HPLC/APCI-MS, chromatography was performed in a similar manner to analytical HPLC except the concentration of acetic acid in the mobile phases was 1%. Mass spectra were recorded using a quadrupole ion trap mass spectrometer (Finnigan LCQ) with the sample being ionised by an APCI source using a vaporiser temperature of 550 °C, sheath and auxillary nitrogen flow pressures of 80 and 20 psi respectively, a capillary temperature of 150 °C and a needle current of 5 μA. The mass spectrometer was controlled by Xcalibur 1.0 software (Finnigan) which was programmed to record the first order mass spectra and then the Collision Induced Dissociation (CID) spectra of three or four of the most intense ions in each first order spectrum by means of the dynamic exclusion facility. CID spectra were obtained by prior isolation of the parent ion in the trap (isolation width 5 amu) and then applying a collision energy of 45% (without wide band activation). Dynamic exclusion allowed the CID spectra of coeluting compounds to be recorded automatically by the instrument without prior knowledge of their molecular masses. Positive ion APCI-MS was used to obtain the pseudomolecular ion of flavonoids, and negative ion APCI-MS (with post-column pre-mass spectrometer neutralization of the acetic acid in the solvent with ammonia) for that of the phenolic acids.

Cell Culture Human dermal fibroblasts or epidermal keratinocytes were isolated from foreskin samples. Cells were maintained in DMEM/10% FCS or Keartinocyte Growth Medium (KGM) and stored in -150 °C freezer. Cells from passage 4 of fibroblasts or passage 3 of keratinocytes were used for the experiments.

MTT Assay for Cell Viability The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] metabolic assay or MTT assay is now widely used to quantify cell proliferation and cytotoxicity. In this study, the MTT assay was used to assess cell damage by the oxidants and cell viability protection by the extract fractions. This method allows a large number of samples at different concentrations to be screened quickly. The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is cleaved to formazan by the succinate-tetrazolium reductase system, which belongs to the mito-

chondrial respiratory chain and is active in living cells. Mitochrondrial electron transport may play a minor role in the cellular reduction of MTT. Most cellular reduction occurs in the cytoplasm and probably involves the pyridine nucleotide cofactors NADH and NADPH. Twenty microliters of solution (5 mg/ml in PBS) was added to each well of 96-well plates and plates were incubated for 2 h at 37 °C. The medium was then removed, and blue formazan was eluted from cells by Hansen's method[17] using 20% SDS in a solution of DMF/water (1 : 1 v/v) at pH 4.3. The plates were shaken on an orbital shaker to solubilize the crystals of formazan. The eluted samples were measured directly in the plate reader at 570 nm (Microplate Manager® 4.0, Bio-Rad Laboratories). Cells were monitored about 1 h after the addition of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] using an Olympus CK 2 microscope.

Assay for Lactate Dehydrogenase (LDH) Release Leakage of LDH enzyme is a well-known marker for cell membrane damage.

$$\underset{\text{NADH+H}^+}{\text{CH}_3\text{–CO–COO}^-} \xrightarrow{\text{LDH}} \underset{\text{NAD}^+}{\text{CH}_3\text{–COOH–COO}^-}$$

The method used here is based on the determination of NADH disappearance using a spectrophotometer set at 340 nm. Decrease in the absorbance at 340 nm is linked directly to the increasing quantity of LDH released by the cells in the culture media. The reaction mixture contained 1.25 ml 0.1 M potassium phosphate buffer (pH 7.4), 0.1 ml 0.25% (w/v) NADH solution, 0.1 ml 1% (w/v) sodium pyruvate solution, and 0.05 ml cell culture supernatant. The disappearance of NADH in the presence of sodium pyruvate was monitored with the Beckman DU-65 series spectrophotometer.[18] The increasing LDH leakage was expressed as decreasing absorbance of each group at 340 nm.

Experimental Design To assess the protective effects of column fractions (A, B, C) on cells, the methods as described before[11,18] to induce cell damage by hydrogen peroxide (H_2O_2) and hypoxanthine–xanthine oxidase (HX–XO) were employed. Human dermal fibroblasts or epidermal keratinocytes were seeded in 96-well or 24-well plates (Iwaki Glass Co.) at high densities in DMEM/10% FCS or KGM, respectively and incubated at 37 °C in a 5% CO_2/95% air atmosphere. Cells were grown to confluence. To investigate the protective or scavenging activities of the fractions, cells were washed with PBS and the different concentrations (50, 100, 200 μg/ml) of fractions A, B and C and H_2O_2 or HX–XO in HBSS (100 or 500 μl/well final volume) was added simultaneously to cells. The controls were optimal and normal cell culture conditions without addition of the fractions or oxidant agents and cells exposed to oxidative damage without presence of the fractions. Cells were incubated for 3 or 7 h, washed twice with PBS, and fresh media were added to the wells. The MTT or LDH release assay was used for the assessment of both cell damage and the effects of the fractions on oxidative damage.

Statistical Analysis Each experiment was performed at least three times. The sets of seven or four wells for the MTT or LDH assay were used to measure effects of the fractions, H_2O_2, HX–XO, or combinations of these compounds. Results were expressed as mean±standard error of the mean of the absorbance. Data were analyzed by one-way analysis of variance (ANOVA) and *post-hoc* analyses after ANOVA employed the Scheffe's test. Differences at the 95% level were considered to be significant.

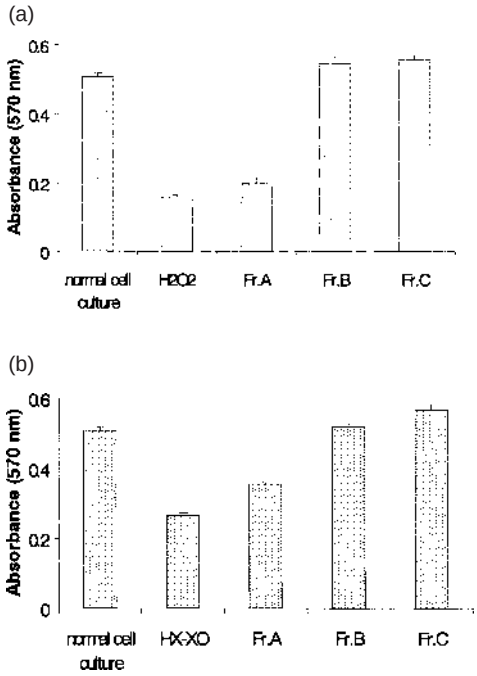

Fig. 2. Protective Effect of the Column Fractions of *C. odorata* Extract on H_2O_2 and HX–XO-Induced Damage to Human Dermal Fibroblasts

Cells were incubated for 3 h at 37 °C with 2×10^{-4} mol/l H_2O_2 (2a) or 2×10^{-2} units/ml XO (2b) with or without fractions. Cells were then washed and assayed by the MTT assay. Bars represent mean±S.E.M. of seven wells. Comparison with cells exposed to generated oxidants with or without fractions is indicated in the graph ($p<0.001$). Fractions B and C show perfect protection on fibroblasts injured by oxidants.

RESULTS

Effects of the Column Fractions (A, B, C) on Fibroblast Injury by H_2O_2 and HX–XO To assess the effects of the fractions on H_2O_2 and HX–XO induced damage, fibroblasts were exposed to H_2O_2 at concentrations of 2×10^{-4} mol/l and to HX–XO of 2×10^{-2} units/ml. These concentrations were found to give significant damage to cells.[11] Fractions A, B, C at 50, 100 or 200 μg/ml were simultaneously added. The concentrations of the fractions B and C at 100 μg/ml showed optimal and complete protection of fibroblasts against H_2O_2 and HX–XO induced damage (Fig. 2a), whereas 50 μg/ml had no effect and 200 μg/ml was similar to 100 μg/ml (data not shown). Fraction A did not show clear protective effect on fibroblasts (Figs. 2a, b).

The fact that fractions B and C were the most effective at 100 μg/ml was reconfirmed by the LDH release assay. Increased absorbance at 340 nm in cells treated by these fractions were interpreted as decreased LDH enzyme leakage by damaged cells (Fig. 3).

Effects of the Column Fractions (A, B, C) on Keratinocyte Injury by H_2O_2 and HX–XO The response of human epidermal keratinocytes to oxidative damage and the protection of extracts were different to that of fibroblasts. All three fractions showed significant protective effects on keratinocytes against H_2O_2 and HX–XO induced damage. Cells

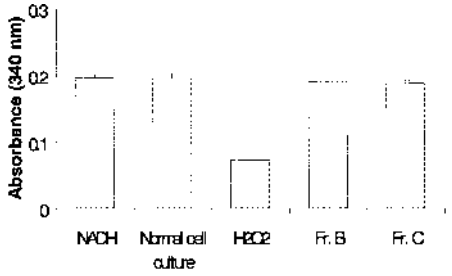

Fig. 3. Protective Effect of Fractions B and C on Hydrogen Peroxide-Induced Damage to Human Dermal Fibroblasts

Cells were incubated for 7 h at 37 °C with 2×10^{-4} mol/l H_2O_2 with or without fractions added. Conditioned media were collected and assayed by LDH release assay. Bars represent mean±S.E.M. of four wells. NADH was used as reference. Comparison with cells exposed to hydrogen peroxide with or without fractions B, C is indicated in the graph. High absorbances at 340 nm of fibroblasts treated with fractions B, C indicate reduced LDH release from H_2O_2 injured cells ($p<0.001$).

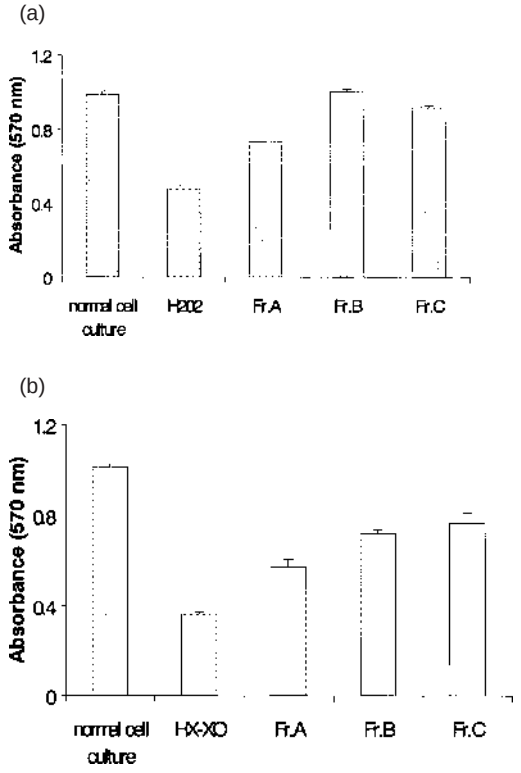

Fig. 4. Protective Effect of the Column Fractions of *C. odorata* Extract on H_2O_2 and HX–XO-Induced Damage to Human Epidermal Keratinocytes

Cells were incubated for 3 h at 37 °C with 2×10^{-4} mol/l H_2O_2 (4a) or 2×10^{-2} units/ml XO (4b) with or without fractions. Cells were then washed and assayed by the MTT assay. Bars represent mean±S.E.M. of seven wells. Comparison with cells exposed to generated oxidants with or without fractions is indicated in the graph. Fractions A, B and C show good or almost complete protection on keratinocytes injured by oxidants ($p<0.001$; 0.05, respectively).

injured by 2×10^{-4} mol/l H_2O_2 were almost 100% protected by the fractions B and C at 100 μg/ml and about 70% by fraction A (Fig. 4a).

When keratinocytes were exposed to HX–XO, the number of survival cells protected by fractions B, C or A was almost 80% or 60%, respectively (Fig. 4b).

Chemical Analysis of the Active Fractions Fractions A, B and C were fractionated further by means of semi-preparative HPLC to facilitate the identification of the compounds present. Fraction A contained a very complex mixture of flavonols, flavanones, flavones and chalcones and a very small amount of phenolic acids. Some flavonoids could be identified by means of chromatographic comparison with standards (HPLC-retention times, UV spectra generated by the diode array detector and mass spectra obtained from the APCI-MS[19]), *e.g.* kaempferide. When no standards were available for comparison, flavonoids could be identified to compound class on the basis of their UV spectra (*e.g.* to flavanones, which have a λ_{max} at *ca.* 290 nm). The number of hydroxyl and methoxyl groups attached to each flavonoid could be deduced from the mass spectra,[19] but not the carbons to which the functional groups are attached to the flavonoid nucleus. For many flavones and some flavonols and chalcones, the substitution patterns can be gathered from the UV spectra, but this does not apply to flavanones, which all tend to have similar UV spectra regardless of substitution. Literature data can be of help in this respect, as a range of flavonoids has already been isolated and identified from *C. odorata.* For instance, the dihydroxy-trimethoxychalcone found in fraction A is likely to be 2′,4-dihydroxy-4′,5′,6′-trimethoxychalcone reported by Barua *et al.*[14] Tamarixetin (=quercetin 4′-methyl ether), kaempferide (=kaempferol 4′ methyl ether), scutellarein tetramethyl ether (=5,6,7,4′-tetramethoxyflavone) and sinensetin (=5,6,7,3′,4′-pentamethoxyflavone) found in fractions A or C have also been reported before from *C. odorata*.[13,14] Eupatilin (=5,7-dihydroxy-6,3′,4′-trimethoxyflavone) and 5-hydroxy-6,7,3′,4′-tetramethoxyflavone have not reported previously. Fractions B and C contained phenolic acids as the major compounds, but also small amounts of flavonoids. A list of the major and the most important minor chemical constituents identified or characterized in the fractions is shown in Table 1, together with the retention times, UV and mass spectra of the compounds.

DISCUSSION

Oxygen radicals or oxidants (hydrogen peroxide, superoxide, hydroxyl radicals) are involved in many human diseases and aging processes. H_2O_2, superoxide (O_2^-) are produced by white cells *via* the myeloperoxidase enzyme or when XO acts on the substrate xanthine and in the presence of oxygen. The latter reaction is seen with ischemia-reperfusion injuries.[20,34] The H_2O_2, O_2^- produced by either white cells or ischemia-reperfusion in the presence of free iron, the Fenton reaction, results in the formation of a hydroxyl (OH) ion, the most potent damaging oxygen radical.[20] In tissue repair, oxidants play an important role in all stages. H_2O_2 was reported to cause fatal injury to fibroblasts, to block cell signaling by inhibition of EGF receptor internalization and to inhibit keratinocyte migration.[21—23]

The O_2^- is also very toxic to tissue and may also result in the generation of free radicals of other types. In chronic wounds such as venous leg ulcers, the ischemia and tissue hypoxia associated with the development of venous ulceration can generate superoxide anions.[24] When a tissue is reperfused following a period of ischemia, large amounts of the superoxide anion are being produced.[24] In addition, the white cells trapped in the skin capillaries also generate these radicals[25] and endothelial cell damage by free radicals with capillary occlusion by neutrophils results in further skin ischemia, which lead to further skin death and breakdown.[26]

Table 1. Major and Minor Constituents of Fractions A, B and C

Purified fraction	Constituents (major ones in bold print)	Compound class	Retention time (min)	UV spectrum (nm)	Molecular mass (*m*/*z*)
A	**Tamarixetin**	Flavonol	19.8	254, 369	316
	Trihydroxymonomethoxyflavanone	Flavanone	19.1	290, 330 sh	302
	Pentamethoxyflavanone	Flavanone	19.3	290, 330 sh	374
	Dihydroxytrimethoxychalcone	Chalcone	23.1	237, 373	330
	Eupatilin	Flavone	21.4	269, 342	344
	5,6,7,4′-Tetramethoxyflavone	Flavone	22.4	265, 322	342
	5-Hydroxy6,7,3′,4′-tetramethoxyflavone	Flavone	22.2	275, 341	358
	Kaempferide	Flavonol	23.2	264, 362	300
	Protocatechuic acid	Hydroxybenzoic acid	4.4	259, 294	154
B	***p*-Coumaric acid**	Hydroxycinnamic acid	11.7	290 sh, 309	164
	***p*-Hydroxybenzoic acid**	Hydroxybenzoic acid	7.1	255	138
	Ferulic acid	Hydroxycinnamic acid	12.3	300 sh, 322	194
	Vanillic acid	Hydroxybenzoic acid	8.1	259, 292	168
	Tetrahydroxymonomethoxyflavanone	Flavanone	17.3	290, 340 sh	318
C	**Protocatechuic acid**	Hydroxybenzoic acid	4.4	259, 294	154
	Vanillic acid	Hydroxybenzoic acid	8.1	258, 293	168
	p-Coumaric acid	Hydroxycinnamic acid	11.7	290 sh, 309	164
	Tetrahydroxymonomethoxyflavanone	Flavanone	17.3	290, 340 sh	318
	Sinensetin	Flavone	20.7	240, 266 sh, 331	372
	Rhamnetin	Flavonol	21.4	255, 371	316

sh denotes shoulder or inflexion.

Natural plasma free-radical scavengers are significantly depleted in the venous leg ulcer or ischemia reperfusion patients compared to the normal control.[26] In thermal injury, oxidants are now considered to be involved in a number of action that can cause severely systemic and local disorders.[30] Oxygen radicals involve in the progression of tissue injury.[31,32] XO and oxygen free radicals were responsible for increased permeability in the pathogenesis of burn edema.[34,35] Anti-oxidant scavenging capacity was also significantly reduced in the local burn wound.[33] It is a relationship between the systemic oxidant level and local burn injury. Early removal of the burned skin after thermal injury significantly diminished the increase in plasma XO activity.[34,35] Studies of the topical application of compounds with free radical scavenging properties on patients or animals have been shown to significantly improve wound healing and protect tissues from oxidative damage.[27—29,36—39]

In this study, H_2O_2 and HX–XO generated O_2^- were employed as an *in vitro* model to cause cultured skin cell injury, in which the protective effect of the *C. odorata* extract fractions were assessed.

In preliminary laboratory investigations we have demonstrated that the crude extract of *C. odorata* contains powerful antioxidants to protect skin cells from oxidative damage.[11] In the present study, the effects of column fractions from the extract on human dermal fibroblasts and epidermal keratinocytes, which are pivotal and crucial in cutaneous wound repair, were further evaluated. H_2O_2 and superoxide radicals generated by the HX–XO reactions were employed as induced oxidative stress to *in vitro* cell culture. The cytotoxicity of the oxidants and protective effects of the extracts were indirectly assessed *via* cell viability. The most active antioxidant compounds were also identified using HPLC and liquid chromatography (LC)-MS technology.

Fractions B and C consistently showed the most protective effects on skin cell culture injured by H_2O_2 and superoxide radicals *in vitro*. Chemical analysis of these fractions (see Table 1) indicated that the major constituents in fraction B were *p*-coumaric acid (*p*-CA=4-hydroxycinnamic acid) and *p*-hydroxybenzoic acid (*p*-HBA=4-hydroxybenzoic acid) and that the major compound in fraction C was protocatechuic acid (PCA=3,4-dihydroxybenzoic acid). Minor compounds in fraction B were ferulic acid (FA=4-hydroxy-3-methoxycinnamic acid) and vanillic acid (VA=4-hydroxy-3-methoxybenzoic acid) and a tetrahydroxy-monomethoxyflavanone. The same flavanone, VA and *p*-CA were minor constituents in fraction C. *p*-HBA is an important natural antioxidant. It is well established as an *in vitro* effective hydroxyl radical scavenger and has Trolox equivalent antioxidant activity (TEAC).[40,41] Other published pharmacological characteristics of *p*-HBA are antibacterial and hypoglycemic properties.[42,43] The presence of *p*-HBA in the extract can account for its bacteria-growth inhibition in human wounds observed previously by clinical researchers.

PCA was detected in fraction C of the extract as a major compound. PCA also is a strongly antioxidant phenolic acid. A number of reports described PCA as a potential chemopreventative agent against carcinogenesis and tumor promotions.[44] PCA isolated form traditional herbal medicines showed strong inhibitory activity against superoxide anion radical ($\cdot O_2^-$).[41] PCA was also found to have similar protective effects as caffeic acid in terms of exhibition of potent protection on cultured endothelial cells against oxidized low-density lipoproteins.[45] In the studies of a polyphenolic extract from *Cudrania cochinchinesis*, another medicinal plant for wound healing, PCA constituted the major constituent and was speculated to be responsible for protection of fibroblasts and endothelial cells against H_2O_2 and HX–XO induced damage.[46]

The *C. odorata* extract also contains a number of hydroxycinnamic acid derivatives. *p*-CA, FA, and VA are present in both fractions B and C. *p*-CA is a major constituent in fraction B. *p*-CA and FA together with caffeic and chlorogenic acids are all hydroxycinnamic acids, which are among the

most widely distributed phenylpropanoids in plant tissues.[40] The biological activities of hydroxycinnamic acid derivatives have been widely investigated. They are potential chemopreventive agents against carcinogenesis and tumor growth.[40] Hydroxycinnamic acid derivatives are also recognized to be strong free radical scavengers to hydroxyl radicals.[40,47]

Hydroxycinnamic acid derivatives were also found to be effective inhibition of XO. The phenolic OH group present in their molecules displays an important contribution to the XO inhibitory activities. The absence of this group in the molecule induces the reduction of the inhibition on XO.[48,49] Other antioxidant activities of hydroxycinnamic acid derivatives have been reported. They were able to scavenge reactive species of oxygen and nitrogen.[51,52] The presence of representatives of two groups of antioxidant phenolic acids (hydroxybenzoic and hydroxycinnamic acids) in fractions B and C can account for their strong inhibition of the cytotoxicity of superoxide radicals and H_2O_2.

Fraction A contained mostly methoxylated flavonoids (flavonols, flavanones, flavones and a chalcone, see Table 1). Although a variety of different flavonoids had already been reported before from *C. odorata*, our present investigations revealed that many more are present in the plant, especially flavanones. Some of the compounds have been demonstrated to be responsible for the hemostatic effect of the *C. odorata* extract.[7] Presence in fraction A of tamarixetin and kaempferide, which are the 4′-methyl ethers of the well-known antioxidant and anti-inflammatory flavonols quercetin and kaempferol, and of small amounts of protocatechuic acid, can explain some of the protective effects on keratinocytes injured by superoxide radicals and H_2O_2. These mixtures of phenolic compounds in the lipophilic fractions of *C. odorata* are likely to be the major contributors of the antioxidant properties of this plant species. In addition, kaempferide has been reported to inhibit inflammation induced by tumor-promoting phorbol esters, and the flavone eupatilin, which is also present in fraction A, selectively inhibits 5-lipoxygenase of cultured mastocytoma cells.[52]

It has been scientifically demonstrated that the extract of *C. odorata* has therapeutic properties in some aspects of wound healing. The results of this study suggest that one of the possible mechanisms to enhanced wound healing by *C. odorata* could be the antioxidant effects of the extracts due to the mixture of the antioxidant phenolics present.

PCA, *p*-HBA, *p*-CA, FA and VA, the major constituents of fractions B and C, are also the main active compounds of many medicinal plants including plants for wound healing, and many biological activities and potential therapeutic applications of these compounds have been reported. Although these compounds are not new, this is the first time, to our knowledge, that these acids were identified in an extract of *C. odorata* and demonstrated to be responsible for the protection of cultured skin cells against oxidative damage. The flavonoids identified in the various fractions are likely to have a synergistic effect.

Crude extract of *C. odorata* has already been used successfully for treating wounds and the present study provides an important scientific basis for understanding how the *C. odorata* extract can benefit wound healing *in vivo*. Clinical efficacy of this plant extract or its preparations should be warranted further by proper, prospective randomized controlled trials. Perhaps a new potential agent for wound healing could be developed from this plant extract.

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