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Phenolic extracts from various Algerian plants as strong inhibitors of porcine liver carboxylesterase

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Abstract
Carboxylesterases (CE), expressed at high levels in human liver and intestine, are thought to detoxify xenobiotics. The goal of this study was to study the effect of phenolic compounds from several plants from the Algerian Atlas used traditionally in Arab folk medicine on the enzymatic activity of porcine liver carboxylesterase. The plants have shown a potent inhibition of carboxylesterase (CE) enzymatic activity in a concentration-dependent manner. Results indicate that the Phenolic extracts from these plants lead to the inactivation of the CE pI = 5.1 with K_i values in micromolar range (1.4–38 μM). These results encourage further biological investigation and identification the inhibitors responsible for this activity.

Keywords: *Medicinal plants,* cleome arabica, *aristolochia* longa, *phenolic compounds, carboxylesterase, inhibition, antioxidant activity*

Introduction

In Arab folk medicine, indigenous plant remedies are widely used in many domains including medicine, nutrition, flavouring, beverage, dyeing, repellents, fragrance, cosmetics, smoking, and other purposes. Recently, interest has increased considerably in finding naturally occurring substances for use in foods or medicines to replace synthetic compounds, which are being restricted due to their carcinogenicity [1].

Many medicinal plants contain large amounts of antioxidants as polyphenols. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as scavenging free radicals and inhibition of lipid peroxidation, are the most important [2,3]. The use of pure polyphénols in medicine has increased to treat many important common diseases, due to their beneficial properties in inhibiting specific enzymes including: hydrolases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases, oxygenase, amino acid oxidases and a number of digestive enzymes including α-amylase, lipase, trypsin and carboxylesterase [4–9].

Carboxylesterases (CE) isoenzymes (EC 3.1.1.1), belonging to the serine esterase family, are widely distributed in different tissues with the highest activity found in hepatic microsomal fractions [10]. These enzymes represent a family of enzymes that hydrolyse functional groups such as carboxylic acid esters (e.g. procaine), amides (e.g. procainamide) and thioesters (e.g. spironolactone) [11,12], as well as catalysing the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, suggesting that carboxylesterases play a physiological role [12]. In addition to hydrolysis, carboxylesterases also catalyse transesterification reactions, fatty acid ester synthesis, clinical prodrug activation and the

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processing of fatty acid and cholesterol derivatives [13,14].

The central biological role of CE appears to be drug and xenobiotic metabolism necessary for the chemo-protective function of proteins in the liver and other front-line tissues [15]. The high specificity of intestinal CE towards exogenous ester-containing substrates along with the presence of the enzyme in the microsomal and cytosolic fractions [16] suggests that the enzyme may be involved in cell signal transduction via diacylglycerol and protein kinase [17,18]. It is therefore important to study the catalytic mechanism responsible for CE-induced hydrolysis, which can be accomplished through the use of potent and selective inhibitors. Also, inhibitors with Ki values in the micromolar range can provide new avenues for the treatment of narcotic abuses and the diseases related to cholesterol [19,20].

The anticancer prodrug 7-ethyl-10-[4-1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) is metabolized by carboxylesterases to the active drug 7-ethyl-10-hydroxycamptothecin and its activation by human intestinal carboxylesterase (hiCE) in the human intestine may contribute to the delayed onset of diarrhoea, a dose-limiting side effect of the drug [21,22].

The purpose of this study is to evaluate the antioxidant activity of a variety of Algerian plants used traditionally for the treatment of digestive system affection, respiratory system troubles, fever, skin disease, rheumatism and other inflammatory diseases, and to examine the inhibitory effects of these phenolic extracts on purified porcine liver CE in order to discover new potential sources of natural enzyme inhibitors.

Material and methods

Materials

10 plants have been evaluated in this study, namely ***Agropyrum repens,***Graminée(aerial part used; medicinal use-depurative, diuretic, emollient, febrifuge, purgative), ***Aristolochia longa,*** Aristolochiacées (roots; astringent, anti-rheumatic, anti-tumor), ***Asteriscus pygmaeus,*** Composées (aerial part; anti-inflammatory, antifongic, insecticide), ***Cléome Arabica,*** Capparidacées (aerial part; diuretic, anti-rheumatic, analgesic), ***Genista Corsica,*** Papilionacées (aerial part; carminative, anti-inflammatory, anti-spasmodic), **Levísticum officinale,** Ombellifères (aerial part; diuretic, carminative, laxative, emmenagogue), ***Malva silvestris,*** Malvacée (aerial part; antiseptic, astringent, bechic, laxative,..), ***Pyganum Harmala,*** Zygophyllacée (seeds; galactagogue, emmenagogue, vermifuge), ***Ruta montana,*** Rutacées (aerial part; tonic, antispasmodic, antiseptic) and ***Salvia verbenaca,*** Officinale (aerial part; stomachic, tonic, vulneraire, anti-rheumatic).

The medicinal plants were gathered in May 2004 from different locations around the town of Laghouat in the steppe region of Algeria. The various data (local name, medicinal uses, used parts of plant, method of preparation and administration) were collected from local inhabitants having knowledge of the curative properties of these plants. The samples were identified at the Agronomic National Institute of Alger, and the voucher specimens were deposited at the laboratory of Fundamental Sciences, University of Laghouat.

All chemicals were purchased from Sigma (USA), Aldrich (Milwaukee,USA), Fluka Chemie (Buchs, Switzerland) and Merck (Germany). Purified porcine liver CE (41 units/mg solid. one unit will hydrolyze 1.0 μmole of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C) was from Sigma- Aldrich Chemie, Germany).

Extraction of phenolics

The dried plant material (2 g) was crushed and extracted for 24 h with 100 mL of 80% (v/v) aqueous methanol at room temperature. The extract was filtered then the residue was extracted for the second time with 50 ml of the same hydrau-alcoholic solvent for 24 h at room temperature. After removal of methanol under reduced pressure in a rotary evaporator at 40°C, the remaining aqueous solution of the extraction was defatted twice with petroleum ether to remove lipids. Then, the lyophilized solution was extracted with ethyl acetate in the presence of an aqueous solution containing 20% ammonium sulphate and 2% of ortho-phosphoric acid solution. The ethyl acetate fraction was dried with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The residue was dried, dissolved in 5 ml of absolute methanol and kept at − 10°C.

Analyses of total phenolic compounds

The amount of total phenolics in the samples was determined with the Folin-Ciocalteu reagent using the method of Lister and Wilson [23]. A standard curve (Figure 1) was obtained using gallic acid as a standard.

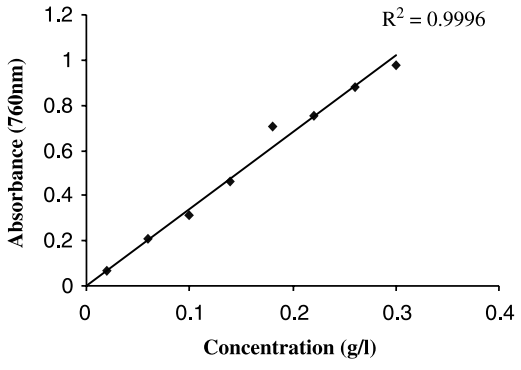

Figure 1. Calibration curve of gallic acid.

Different concentrations of gallic acid were prepared in distilled water, and their absorbances were recorded at 765 nm. 100 μL of sample was added to 500 μL (1/10 dilution) of the Folin-Ciocalteu reagent. The solutions are mixed and incubated at room temperature for 2 min and then 2000 μL of 20% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 30 min in the dark at room temperature. The absorbance of all samples was measured at 760 nm using a Milton Roy 601 UV–visible spectrophotometer and the results are expressed in mg of gallic acid per g (GEA) of dry weight of plant.

Estimation of flavonoids content

The flavonoids content in the extracts was determined spectrophotometrically according to Lamaison and Carnat [24] using a method based on the formation of the complex flavonoids-aluminium, having an absorption maximum at 430 nm. Rutin was used for the calibration curve. 1 mL of diluted sample was separately mixed with 1 mL of 2% aluminum chloride methanolic solution. After incubation at room temperature for 20 min, the absorbance of the reaction mixture was measured at 430 nm with a Milton Roy 601 UV–visible spectrophotometer and the flavonoids content is expressed in mg per g rutin equivalent (RE) of dry weight material.

Quantification of flavonols content

The content of flavonols was determined by the literature method [25]. Quercetin was used for the calibration curve. 2 mL of diluted sample was added to 2 mL (20 g/l) aluminum trichloride and 6 mL (50 g/l) sodium acetate solutions. The absorption at 440 nm was read after 2.5 h at room temperature using a (UV-1601) UV-Visible spectrophotometer and the flavonol content was calculated in mg per g quercetin equivalent (QE) of dry material.

Evaluation of antioxidant activity (DPPH assay)

Radical scavenging activity of plant extracts against stable $DPPH^{\cdot}$ (2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically. When $DPPH^{\cdot}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 517 nm on a UV-visible light spectrophotometer (UV-1601) by the slightly modified method of Brand Williams et al. [26], as described below. The solution of $DPPH^{\cdot}$ in methanol (500μM) was prepared daily, before measurements. Various concentrations of 1 mL of sample solution diluted in Tris buffer solution (100 mM; pH = 7.4) were added to 1 mL of the $DPPH^{\cdot}$ radical solution. The mixture was then shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorption was measured at 517 nm. Absorption of a blank sample containing the same amount of buffer and $DPPH^{\cdot}$ solution was prepared and measured daily. The antioxidant activity of the extract was expressed as an IC_{50} value defined as the concentration (in μM) of the extract that inhibited the formation of DPPH radicals by 50%. The DPPH radical scavenging activity obtained for each plant extract was compared with that of Trolox, an analog of vitamin E.

Enzyme inhibition assay

Determination of *K*i values was performed for CE using *p*-nitrophenyl acetate as substrate. Inhibition constants were calculated by assessment of the reduction in the formation of *p*-nitrophenol, monitored by a spectrophotometric assay at 414 nm at 37°C in a total volume of 2500 μl of 20 mM (Tris, pH = 7.4), and data were recorded at 15 s intervals for up to 5 min. Carboxylesterase specific activity was expressed as μmol/min/mg protein using four concentrations of substrate (0.025–0.1 mM). Enzyme velocity values (1/v) versus inhibitor concentrations were plotted and *K*i values were calculated from the curve.

Results and discussion

The phenolic extracts of ten plants known for their therapeutic properties in traditional Arab medicine were tested for their antioxidant status and inhibitory activity towards purified porcine liver CE. The total phenol content of each fraction was estimated by the Folin–Ciocalteu procedure, and the amount of polyphenols in the plants was expressed in milligrams per gram of dry matter.

Antioxidant activity by the DPPH method and total phenolic content

So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts. Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics [27]. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties and enzyme inhibition. The former properties are especially distinctive for flavonols, so, therefore, the content of both groups of phenolics was determined in the extracts (Table I).

The content of phenolic compounds (mg/g) in the methanolic extracts, determined from the calibration curve (Figure 1) and expressed in gallic acid equivalents (GAE), varied between 1.15 and 7.2.

Table I. Total amount of plant phenolic compounds, flavonoids and flavonols.

Name of Plant	Total phenolics (mg GAE/g dw)[a]	Flavonoids content (mg RE/g dw)[b]	Flavonols content (mg QE/g dw)[c]
Salvia verbenaca	7.2 ± 0.04	3.04 ± 0.01	0.85 ± 0.001
Ruta Montana	5.63 ± 0.01	2.07 ± 0.03	0.01 ± 0.0015
Genista Corsica	4.73 ± 0.02	4.61 ± 0.01	4.39 ± 0.005
Malva silvestris	2.78 ± 0.04	2.02 ± 0.06	0.01 ± 0.002
Asteriscus pygmaeus	2.77 ± 0.03	1.11 ± 0.08	0.68 ± 0.008
Levísticum officinale	2.08 ± 0.01	1.16 ± 0.05	0.75 ± 0.005
Pyganum Harmala	2.01 ± 0.06	0.52 ± 0.02	0.11 ± 0.002
Agropyrum repens	1.64 ± 0.03	0.39 ± 0.05	0.003 ± 0.0001
Aristolochia longa	1.47 ± 0.02	0.81 ± 0.02	0.41 ± 0.002
Cléome Arabica	1.15 ± 0.05	1.01 ± 0.08	0.93 ± 0.008

[a]milligrams of gallic acid equivalent per gram of dry weight of plant.[b]milligrams of rutin equivalent per gram of dry weight of plant.[c] milligrams of quercetin equivalent per gram of dry weight of plant.

The highest amounts were found in the extracts of *Salvia verbenaca* and *Ruta montanas*, The amount of total phenolic compounds in all the tested plants is less than that in some Algerian medicinal plants [28].

The content of flavonoids (mg/g), in rutin equivalents varied from 0.39 to 4.61. The highest amounts of flavonoids were found in extracts of *Genista Corsica, Malva silvestris* and *Cleome arabica* and the other plants examined contained remarkably lower amounts of these compounds. Relatively low amounts of flavonoids were also determined in *Agropyrum repens* which contained 40.93% of these compounds as a fraction of the total phenolics.

The concentration of flavonols, expressed in quercetin equivalents in mg/g of plant extract, varied widely as compared with the total phenolics and flavonoids, i.e. from 0.003 to 0.93. In general, some correlation between total phenolics and flavonols can be observed, although the highest amounts were found in *Genista Corsica,* the plant with a medium concentration of phenolics.

Phenolic compounds are known as powerful chain-breaking antioxidants [29,30] and are very important plant constituents because of their scavenging ability due to their hydroxyl groups [31]. It has been suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested at up to 1 g daily from a diet rich in fruits and vegetables [32].

The general free radical scavenging activity of the extract was evaluated by its interaction with DPPH in solution. DPPH$^{\cdot}$ is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule and because of its odd electron, the methanolic solution shows a strong absorption band at 517 nm [33], which decreases in the presence of free radical scavengers. This scavenging activity has been largely used as a quick and reliable parameter to assess the in vitro general antioxidant activity of plant extracts [34–36], especially that assigned to phenolic compounds i.e phenolic acids and flavonoids [37,38].

In the current study, the scavenging activities of DPPH exerted by our phenolic extracts as well as that of Trolox, were calculated from the linear % inhibition–concentration curves. The concentration of inhibitors was calculated in μM using an average molecular weight for polyphenols of 500 g. The results are summarized in (Table II).

All the plant extracts showed a beneficial effect against free-radical damage compared to the standard antioxidant, Trolox. *Cléome Arabica, Asteriscus pygmaeus and Salvia verbenaca* were the best inhibitors and their inhibitory effects were almost 5-fold more potent than that of Trolox which is classed as a potent standard antioxidant [39]. *Genista Corsica* and Levísticum officinale had significant potency and were 2-fold more active as the standard. Other extracts had a weaker activity than that of Trolox.

It can be observed that the phenolic content of the extracts had no significant correlation with their antiradical activity as measured by the DPPH assay ($R^2 < 0$). Moreover, the amount of flavonoids in the analysed plant extracts showed only low correlation with the total amount of phenolics

Table II. Free radical (DPPH) scavenging activity of the 10 medicinal plant extracts.

Name of Plant	IC_{50} (μM)*
Cléome Arabica	13.24 ± 0.1
Asteriscus pygmaeus	14.97 ± 0.1
Salvia verbenaca	16.92 ± 0.2
Genista Corsica	25.05 ± 0.1
Levísticum officinale	39.86 ± 0.3
Pyganum Harmala	57.98 ± 0.3
Ruta Montana	62.77 ± 0.2
Agropyrum repens	63.33 ± 0.2
Malva silvestris	86.49 ± 0.3
Aristolochia longa	90.16 ± 0.4
Trolox	72.63 ± 0.3

*IC_{50} value represents the concentration in micro-molar of the phenolic extracts per litre of the mixture that scavenge DPPH radical by 50%. Trolox was used as a standard antioxidant.

($R^2 = 0.24$) and also with radical scavenging activity (correlation coefficient between data of flavonoid content and DPPH assay with methanolic extracts was only 0.08). It is known that only flavonoids of a certain structure and particularly the hydroxyl position in the molecule determine antioxidant properties; in general these properties depend on the ability to donate hydrogen or an electron to a free radical. In our study flavonols, important compounds in terms of radical scavenging properties, had a weaker correlation between their content and the antiradical activity of plant extracts as compared to flavonoids ($R^2 = 0.03$). A detailed examination of the phenolic composition of the plant extracts is required for a comprehensive assessment of the individual compounds exhibiting antioxidant activity.

The results of this study show that these plants can be used as an easily accessible source of natural antioxidants either as a possible food supplement or in the pharmaceutical industry. However, the components responsible for the antioxidant activity of these plants are currently not known and further work should be performed on their isolation and identification.

Inhibition of porcine liver CE (in the 4.5–6.5 pI range)

In the present study we have been interested in the evaluation of the phenolic extracts capacities to inhibit carboxylesterase porcine liver activity (CER) (EC.3.1.1.1). where the enzymatic activity of CER was titrated using *p*-nitrophenylacetate as substrate (PNPA) which releases *p*-nitrophenol with a detectable yellow colouring.

CER showed Michaelis kinetics using p-nitrophenyl acetate (PNPA) as substrate and the kinetic parameters of the enzyme (V_m, k_{cat} and k_m) were calculated using a Lineweaver Burk plot [$1/v = f\ (1/S)$] (Figure 2) and the results are given in Table III.

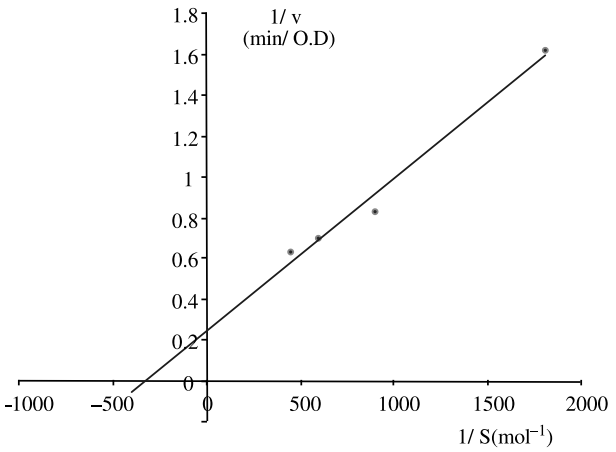

Figure 2. Lineweaver-Burk graphical representation of the enzymatic kinetics of CE.

Table III. kinetic parameters of carboxylesterase on the PNPA.

[E] (μM)	V_M [ΔDO/min]	K_M (mM)	K_{CAT} (min^{-1})	K_{CAT}/K_M $min^{-1}.mM^{-1}$
0.009	4.198	2.94	25350.24	8622530.61

Inhibition constants, *K*i, for each extract were estimated by a Dixon plot (Figure 3), the phenolics concentration being expressed in molar equivalents (μM) of gallic acid. The influence of incubation time on the inhibitory activity by polyphenol fractions was tested up to 10 min, but preincubation of CE with the fractions did not significantly cause a time-dependent inhibition. All plant phenolic extracts were found very significantly to inhibit CE activity, in a concentration-dependent manner. The best inhibitors were the phenolic extracts of *Cleome Arabica* and *Aristolochia longa*. To investigate the type of enzyme inhibition, CE activity was assayed in the presence of different concentrations of the substrate ([PNPA] $< 10\,K_m$) [40] (0.25–1.0 mM) and polyphenol extracts. The *K*i value was obtained from a Dixon [41] plot which

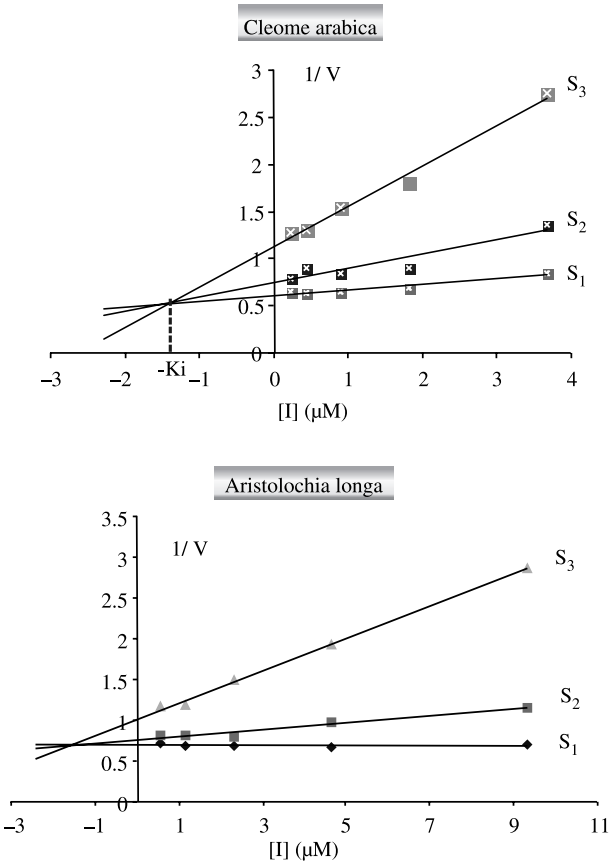

Figure 3. Dixon plots of ihibition of porcine liver carboxylesterase according to the total phenol concentration of *Aleome arabica* and *Aristolochia longa* plants. Activities were determined by formation of PNPA by CE at several substrate concentrations: 0.025 mM (S_1), 0.05 mM (S_2) and 0.1 mM (S_3). The points represent the means of three experiments.

Table IV. *Ki* and inhibitor type for some total polyphenol extracts obtained for commercial porcine CE ($E = 0.009\,\mu M$).

Name of plant	*Ki* (μM)*	Type of inhibition
Cléome Arabica	1.4 ± 0.01	*Competitive*
Aristolochia longa	1.6 ± 0.02	*Competitive*
Levísticum officinale	3.0 ± 0.1	*Competitive*
Agropyrum repens	3.1 ± 0.2	*Competitive*
Pyganum Harmala	3.9 ± 0.1	*Competitive*
Ruta Montana	4.8 ± 0.2	*Competitive*
Asteriscus pygmaeus	8.7 ± 0.3	*Competitive*
Malva silvestris	9.0 ± 0.3	*Competitive*
Genista Corsica	11.0 ± 0.5	*Competitive*
Salvia verbenaca	38.0 ± 0.4	*Competitive*

*phenolics concentration expressed in molar equivalents (μM) of gallic acid that inhibit 50% of the CE activity.

showed that the inhibition by all the investigated plants was competitive with *K*i values 1.4–38 μM (see Table IV). *Salvia verbenaca* was least potent with a *K*i value of 38 μM.

We sought to elucidate the relationship between the general structural type of the phenolic compounds and inhibitory potency, having spectrophotometrically quantified the flavonoid and flavanol class. Our results highlighted that extracts containing flavonoid derivatives, specially flavonol, showed potent CE inhibitory activity in some plant extracts, while extracts containing a relatively high percentage of hydroxycinnamic and hydroxybenzoic derivatives exhibited a weak inhibitory effect.

The correlation between *Ki* values and total phenolic contents of Algerian plants had a correlation coefficient of $R^2 = 0.70$ (Figure 4). This result suggests that the inhibitory activity was not proportional to the concentration of phenolic compounds. Also, it may be that the inhibitory potency of the plant extracts is not limited to the phenolic content but may be due to the presence of some individual active phenolic compounds. Thus the

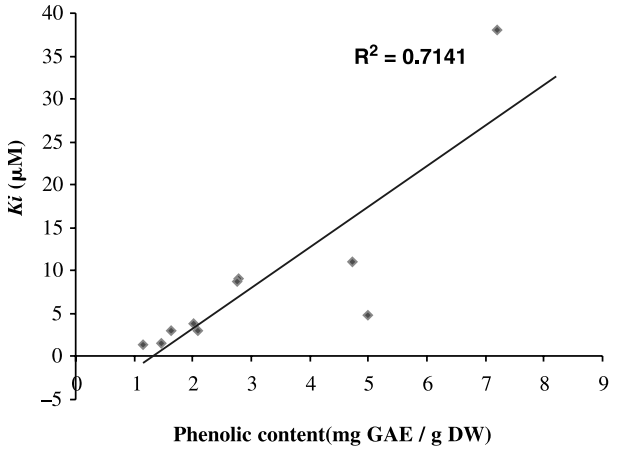

Figure 4. Relationship between the inhibitor concentration *Ki* of *CE* and total phenolic contents of the 10 plants.

inhibitory potency of an extract cannot be explained just on the basis of its phenolic content but also requires their proper characterization. There are several reasons to explain the ambiguous relationship between inhibitory potency and total phenolics: total phenolics content did not include all the possible inhibitors; the synergism among the inhibitors in the mixture accounted for the inhibition, but was not only dependant on the concentration of individual inhibitors, but also on the structure and interaction among them. Consequently, *Aristolochia longa* and *Agropyrum repens*, with similar concentrations of total phenolics may vary in their inhibitory activity (1.16 and 3.1 μM respectively).

The results suggested that the phenolic compounds of the medicinal plants contributed significantly to the inhibition of CE activity. Although there was no significant correlation found between the antioxidant activity and *Ki* values. During the screening of ten plants in this work, *Cleome Arabica was* found to be the most promising. This plant which contained the lowest amount of phenolics (1.15 mg/g DW) had a very high level of antioxidant activity and was a potent inhibitor of CE enzymatic compared to the other plants.

In our previous work, we have tentatively identified phenolics as a class of compounds present in the plant extracts of all the plants species we investigated which showed a potent inhibitory activity towards CE [42]. In this study, it is difficult to be conclusive about the most active class of phenolic compounds and the observed effect of *Cleome Arabica* and *Aristolochia longa* extracts is probably due to the direct effect of some individual phenolic compounds. In summary, our data show that the phenolic extracts of all medicinal plants, especially *Cleome Arabica* and *Aristolochia longa*, lead to inactivation of the CE pI = 5.1 specifically, with *K*i values of 1.4 and 1.6 μM, respectively. CEs have been showed to hydrolyse phorbol esters which cause various effects in different tissues in different species by activation of protein kinase C [43,44]. Since protein kinase C has been suggested to be involved in carcinogenesis and cell proliferation [45,46], it is important that changes in CE due to exogenous compounds with phorbol-hydrolyzing activity should be clarified. We suggest that phenolics, including those especially contained in the extracts of *Cleome Arabica and Aristolochia longa* with CE-inhibitory properties, may be able to regulate the enterocyte cellular expression via biochemical mechanism related to signal transduction.

The phenolic compounds present in these plants may also serve as lead compounds for the synthesis of a series of inhibitors. In several cases, the use of the plants and their respective therapeutic prescription in popular medicine are not easy to understand, and we can question if there is a relation between the therapeutic properties of these plants and their inhibitory effects on CE activity. Although the

so-called polyphenols remain to be separated and their structure elucidated, and their mechanism of action at the molecular level remains to be elucidated, this work is the first report on the inhibitory activity of the pI = 5.1 CE by phenolic compounds, which may explain the beneficial folk medical utilisation of these plants for the treatment of some intestinal disorder and further work is required.

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