Inhibition of Thyroid Peroxidase by Myrcia uniflora Flavonoids

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Research Section

Thyroid Peroxidase Inhibition by *Kalanchoe brasiliensis* Aqueous Extract

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Abstract—Flavonoids are known inhibitors of thyroid peroxidase (TPO) and some are components of *Kalanchoe brasiliensis*, a plant used in popular medicine to treat tissue injuries, enlarged ganglia and peptic ulcer. As *K. brasiliensis* extract is currently used, the present study was designed to evaluate the effects of *K. brasiliensis* aqueous extract on TPO activity. We show here that TPO iodide-oxidation activity was significantly inhibited by *K. brasiliensis* aqueous extract and that TPO inhibition seems to be competitive, since the enzyme V_{max} was unchanged and K_m for iodide was significantly increased in the presence of the plant extract. Furthermore, TPO inhibitory activity produced by *K. brasiliensis* extract was unchanged after boiling or by incubation with hepatic enzymes (activated S9 fraction), suggesting that at least the antithyroid component of this plant infusion could probably reach systemic circulation. We also report that *K. brasiliensis* aqueous extract is able to scavenge H_2O_2, *in vitro*. As H_2O_2 is an essential TPO cofactor, it is possible that the H_2O_2 trapping effect of *K. brasiliensis* may be responsible, at least in part, for the inhibition of the iodide-oxidation reaction catalysed by this enzyme. Thus, *K. brasiliensis* aqueous extract has antithyroid effects *in vitro*, suggesting that its chronic consumption could contribute to the development of goitre and hypothyroidism, mainly in areas of low iodine intake. © ***2000 Elsevier Science Ltd. All rights reserved***

Keywords: thyroid peroxidase; *Kalanchoe brasiliensis*; hydrogen peroxide.

Abbreviations: MMI = methimidazole; PTU = propylthiouracil; TPO = thyroid peroxidase.

INTRODUCTION

Thyroid peroxidase (TPO) is a heme-containing glycoprotein bound to the apical membrane of thyroid cells which, in the presence of H_2O_2, catalyses iodide oxidation, iodination of tyrosyl residues and coupling of iodotyrosines in the thyroglobulin molecule, leading to the formation of thyroid hormones (Taurog, 1996).

Flavonoids are widely distributed in plant-derived foods that have a variety of biological activities including antioxidant (Middleton, 1984) and antithyroid (Cody *et al.*, 1989; Divi and Doerge, 1995; Doerge and Divi, 1995; Lindsay *et al.*, 1989) effects. It has previously been reported that the consumption of flavonoids and other xenobiotics by experimental animals reduced both thyroid iodide ion uptake and TPO activity, producing enlargement and histological changes in the thyroid gland (Cody *et al.*, 1989; Lindsay *et al.*, 1989; Moudgal *et al.*, 1958).

Kalanchoe brasiliensis Camb. (Crassulaceae) is a Brazilian medicinal herb traditionally employed to treat injuries, abscesses, enlarged ganglia, and inflammatory processes (Lucas and Machado, 1946; Rossi-Bergmann *et al.*, 1997). It is widely used in Brazil's popular medicine as an aqueous extract, and is known to contain many flavonoids (Rossi-Bergmann *et al.*, 1997). However, there are only few studies about the pharmacological effects of this plant, and there seems to be no information about the possible antioxidative or antithyroid capacity of *K. brasiliensis*. So the aim of this study was to determine whether *K. brasiliensis* produces *in vitro* effects, similar to those of the generally used antithyroid drugs, the thioureylene compounds: propylthiouracil and methimazole, which act as TPO inhibitors *in vivo* and *in vitro* (Taurog, 1996). Futhermore, because of the antioxidant effect of

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some flavonoids (Middleton, 1984), we also studied the H_2O_2 scavenging ability of *K. brasiliensis* aqueous extract, since H_2O_2 is necessary for normal TPO activity.

MATERIALS AND METHODS

TPO preparation

TPO was extracted from human diffuse toxic goitre tissue samples obtained during thyroidectomy (informed consent given by patients), as described by Moura *et al.* (1989) and Carvalho *et al.* (1994). After cleaning, on an ice-cooled glass plate, the thyroid tissue samples (1 g) were minced and homogenized in 3 ml 50 mM Tris–HCl buffer, pH 7.2, containing 1 mM KI, using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 100,000 ***g***, 4°C for 1 hr. The pellet was suspended in 2 ml digitonin (1%, w/v) and incubated at 4°C for 24 hr to solubilize the peroxidase. The digitonin-treated suspension was centrifuged at 100,000 ***g***, 4°C for 1 hr, and the supernatant containing the solubilized TPO was used for the assays.

Plant extract

Kalanchoe brasiliensis leaves were washed and then macerated in distilled water (20%, w/v), followed by heating (50–60°C) for 30 min. The extract was filtered twice and the sediment-free filtrate (20% aqueous extract) was collected and diluted in 50 mM sodium phosphate buffer, pH 7.4, before assays.

TPO iodide-oxidation activity inhibition

The TPO iodide-oxidation activity was measured as previously described (Carvalho *et al.*, 1994; Moura *et al.*, 1989; Nakashima and Taurog, 1978; Pommier, 1978). The control assay mixture contained 1.0 ml of freshly prepared 50 mM sodium phosphate buffer, pH 7.4, containing 24 mM KI and 11 mM glucose, and the amount of solubilized TPO producing an iodide oxidation activity of 0.1 $\Delta A_{353\ nm}$/min. The final volume was adjusted to 2.0 ml with 50 mM sodium phosphate buffer, pH 7.4, and the reaction was started by the addition of 10 μl 0.1% glucose oxidase (Boehringer Grade I). The increase in absorbance at 353 nm (tri-iodide production) was registered for 4 min on a Hitachi spectrophotometer (U-3300). In order to test the inhibitory effects, the desired amounts of *K. brasiliensis* extract, propylthiouracil (PTU) or methimazole (MMI) in 50 mM sodium phosphate buffer, pH 7.4, were added to the assay mixture before adjusting the final volume to 2 ml. The $\Delta A_{353\ nm}$/min in the presence or absence of inhibitors was determined from the linear portion of the reaction curve.

The inhibitory activity was expressed as the concentration necessary to produce a 50% inhibition of the original peroxidase activity (IC_{50}). Each compound was tested in at least three series of experiments, in which eight to 12 different concentrations were assayed.

Iodide oxidation inhibitory kinetics

In order to evaluate the kinetic parameters of TPO-catalysed iodide-oxidation inhibition, a given TPO activity was assayed as described above, with or without 0.07% *K. brasiliensis* extract (IC_{50}) and variable iodide concentrations. Each iodide concentration was tested three times in the presence or absence of *K. brasiliensis* aqueous extracts, and the $\Delta A_{353\ nm}$/min obtained were plotted against KI concentrations.

H_2O_2 trapping effect

To study whether *K. brasiliensis* is able to scavenge H_2O_2, 4.0 μM H_2O_2 (Merck) was incubated in the presence or absence of 0.07% (IC_{50}) and 0.8% (IC_{100}) *K. brasiliensis* extract. Aliquots of 100 μl were then added to 1 ml 0.2 M sodium phosphate buffer, pH 7.8, containing scopoletin (5.0 μM) and horseradish peroxidase (5 μg/ml). The fluorescence was measured in a Hitachi (F4000) spectrofluorometer (excitation = 360 nm, emission = 460 nm), as previously described (Déme *et al.*, 1985). The fluorescence measurements were plotted against H_2O_2 concentrations.

In vivo, the thyroid gland generates H_2O_2 gradually, so an enzymatic system (glucose-glucose oxidase) was used as a model to test the effect of *K. brasiliensis* extract on maintained production of H_2O_2. *K. brasiliensis* extract (0.07%) was incubated in the presence of 11 mM glucose and the final volume was adjusted to 2.0 ml with 50 mM sodium phosphate buffer, pH 7.4. The reaction was started by the addition of 10 μl 1 mg/litre glucose oxidase (Boehringer Grade I). This concentration of glucose oxidase produces an *in vitro* H_2O_2 generation similar to that produced by the porcine and the human thyroid NADPH-oxidase, the enzyme responsible for H_2O_2 production *in vivo* (Leseney *et al.*, 1998). 100-μl aliquots of the reaction mixture were transfered to test tubes 0, 5, 10 and 15 min after glucose oxidase addition. Then, 1 ml scopoletin solution was added and the fluorescence was measured as above. The H_2O_2 produced, proportional to scopoletin fluorescence decrement, was plotted against time.

Heat effect

TPO iodide-oxidation inhibitory activity was measured as described above using *K. brasiliensis* aqueous extracts heated to 100°C for 30 min.

Hepatic microsomal enzymes treatment

In order to test the effects of hepatic microsomal enzymes on the TPO inhibitory activity of *K. brasiliensis* aqueous extracts and thioureylenes, different concentrations of these compounds were incubated

in a final volume of 1 ml, with or without 0.4 ml activated S9 fraction (8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP and 1.6 mg hepatic S_9 fraction protein/ml in 0.1 M sodium phosphate buffer, pH 7.4) (Maron and Ames, 1983), at 37°C for 30 min. The reaction was stopped by heating to 100°C for 10 min. The activated S9 fraction alone was used as control in the inhibitory assays.

RESULTS

TPO iodide-oxidation activity was significantly inhibited by *Kalanchoe brasiliensis*, with a 50% inhibition of the original TPO activity (IC_{50}) obtained at a 0.07% *K. brasiliensis* aqueous extract concentration (Fig. 1). The TPO activity inhibition produced by thioureylenes, PTU and MMI, well-known antithyroid agents that act through TPO inhibition *in vivo* and *in vitro*, were evaluated in comparison. The already described concentrations of PTU and MMI necessary to produce a 50% inhibition of TPO-mediated thyroglobulin iodination were 19.5 μM and 10 μM, respectively (Taurog, 1996). Under our experimental conditions we have found similar differences in the IC_{50} values for the PTU (9.8 μM) and MMI (3.8 μM) inhibitory effects on the TPO iodide-oxidation reaction. Hence, our TPO assay system can be compared with those reported by other authors, at least as related to PTU and MMI effects.

The possibility of a TPO inhibition caused by competition with the substrate (iodide) was evaluated. Kinetic iodide-oxidation studies show that in the presence of *K. brasiliensis* aqueous extract TPO $K_{0.5}$ for iodide was significantly increased (without *K. brasiliensis*: 11.4 ± 2.3 mM; with *K. brasiliensis*: 27.8 ± 4.8 mM, $P < 0.05$), but not V_{max} (without *K. brasiliensis* = 0.22 ± 0.02 $\Delta A_{353\ nm}$/min; with *K. brasiliensis*: 0.25 ± 0.05 $\Delta A_{353\ nm}$/min) (Fig. 2).

To further evaluate the possible mechanism of TPO inhibition by *K. brasiliensis* aqueous extracts, we tested whether it possessed H_2O_2 scavenging effect. Our results show that *K. brasiliensis* significantly scavenges H_2O_2 added to the incubation mixture (Fig. 3) or H_2O_2 generated by the glucose–glucose oxidase system (Fig. 4).

As *K. brasiliensis* leaves are frequently consumed as tea, we also tested the anti-TPO activity of aqueous *K. brasiliensis* after boiling. The concentration of the boiled preparation necessary to produce a 50% inhibition of peroxidase activity (IC_{50}) was similar to the IC_{50} found using non-boiled aqueous extract.

Furthermore, hepatic microsomal enzymes (activated S9 fraction) had no effect on the TPO inhibitory activity of *K. brasiliensis* aqueous extracts and thioureylenes, since neither *K. brasiliensis* nor

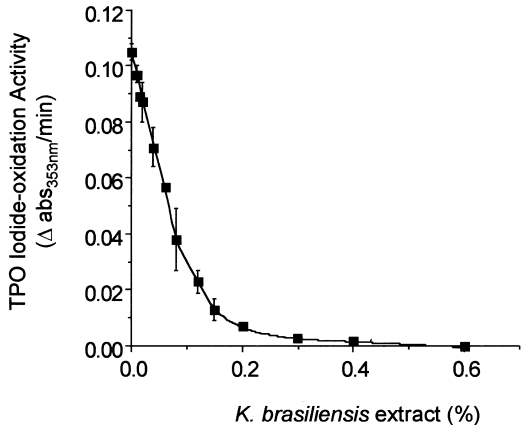

Fig. 1. TPO iodide-oxidation inhibitory activity produced by *Kalanchoe brasiliensis* aqueous extract. The TPO iodide-oxidation activity was measured in the presence of different *K. brasiliensis* aqueous extract concentrations, as follows: the amount of solubilized TPO producing a fixed iodide oxidation activity ($\Delta A_{353\ nm}$/min = 0.1) was assayed in the presence of 1.0 ml freshly prepared 50 mM sodium phosphate buffer, pH 7.4, containing 24 mM KI, 11 mM glucose and *K. brasiliensis* extract in a final volume of 2 ml. The reaction was started by the addition of 10 μl 0.1% glucose oxidase. The increase in absorbance at 353 nm was followed for 4 min on a computerized Hitachi spectrophotometer (U-3300). The iodide-oxidation activity ($\Delta A_{353\ nm}$/min) was determined from the linear portion of each reaction curve and plotted against different *K. brasiliensis* aqueous extract concentrations. Results are expressed as mean ± SEM.

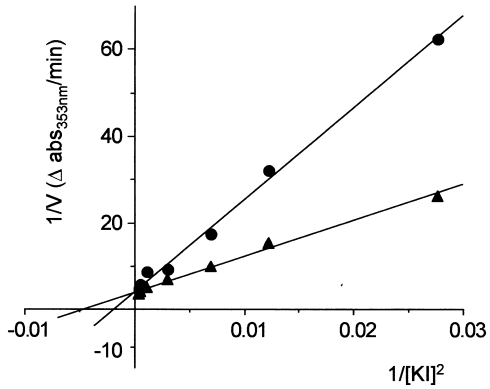

Fig. 2. Iodide concentration dependence of the TPO-catalysed iodide-oxidation inhibitory activity produced by *K. brasiliensis*. The amount of solubilized TPO producing a fixed iodide oxidation activity ($\Delta A_{353\ nm}$/min = 0.1) was assayed in the presence (●) or absence (▲) of 0.07% *K. brasiliensis* extract (IC_{50}). Different concentrations of KI were added and the final volume was adjusted to 2.0 ml. The reaction was started by the addition of 10 μl 0.1% glucose oxidase. The increase in absorbance at 353 nm ($A_{353\ nm}$) was followed for 4 min on a computerized Hitachi spectrophotometer (U-3300). The iodide-oxidation activity ($\Delta A_{353\ nm}$/min) was determined from the linear portion of each reaction curve and plotted against different iodide concentrations. Each iodide concentration was tested three times in the presence or absence of *K. brasiliensis* aqueous extracts, and the $\Delta A_{353\ nm}$/min obtained were plotted against KI concentrations. A representative experiment is shown.

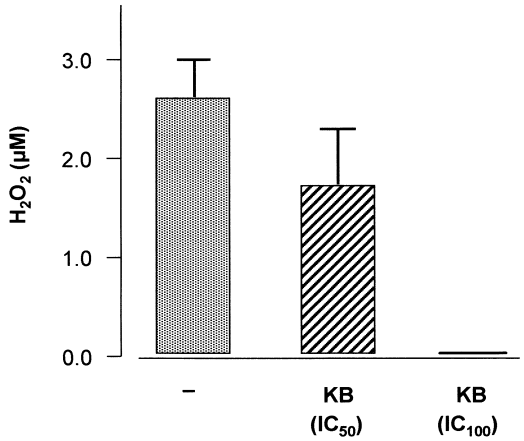

Fig. 3. H_2O_2 trapping effect of *K. brasiliensis* aqueous extract. H_2O_2 concentration was measured after incubation with or without *K. brasiliensis* extract (KB), as follows: 4.0 μM H_2O_2 was incubated in the presence or absence of 0.07 % (TPO–IC_{50}) or 0.8 % (TPO–IC_{100}) *K. brasiliensis* extract. Aliquots of 100 μl were transferred to a tube and then 1 ml 0.2 M sodium phosphate buffer, pH 7.8, containing scopoletin (5.0 μM) and horseradish peroxidase (5 μg/ml) was added. The fluorescence was measured in a Hitachi (F4000) spectrofluorometer (excitation = 360 nm, emission = 460 nm). Results are expressed as mean ± SEM.

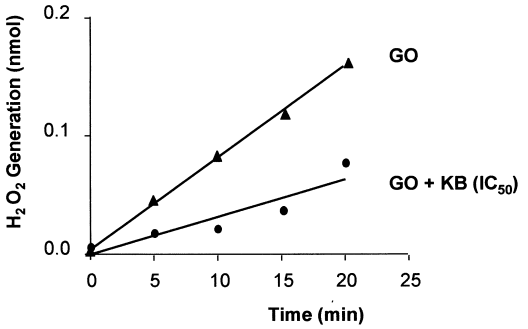

Fig. 4. Effect of *K. brasiliensis* extract on H_2O_2 produced by glucose-glucose oxidase enzymatic system. Glucose (11 mM) was incubated in the presence (●) or absence (▲) of *K. brasiliensis* aqueous extract (KB; 0.07%) and the final volume was adjusted to 2.0 ml with 50 mM sodium phosphate buffer, pH 7.4. The reaction was started by the addition of 10 μl 1 mg/litre glucose oxidase (GO; Boehringer Grade 1). Aliquots of 100 μl were transferred to the test-tube 0, 5, 10 and 15 min after glucose oxidase addition. Then, scopoletin solution (1 ml 0.2 M sodium phosphate buffer, pH 7.8, containing 5.0 μM scopoletin and 5 μg horseradish peroxidase/ml) was added. The fluorescence was measured in a Hitachi (F4000) spectrofluorometer (excitation = 360 nm, emission = 460 nm). H_2O_2 concentrations were plotted against time. Results are expressed as mean of two different experiments.

thioureylenes IC_{50} were changed by treatment with hepatic microsomal enzymes.

DISCUSSION

Kalanchoe brasiliensis aqueous extract inhibits TPO iodide-oxidation activity competitively, since TPO $K_{0.5}$ for iodide was significantly increased in the presence of *K. brasiliensis* aqueous extract but not V_{max} (Fig. 2).

It is quite possible that the inhibition of TPO iodide-oxidation reaction produced by *K. brasiliensis* could be due both to a direct effect on TPO activity and to its ability to scavenge H_2O_2, since *K. brasiliensis* significantly scavenges H_2O_2 added to the incubation mixture (Fig. 3) or H_2O_2 generated by the glucose–glucose oxidase system (Fig. 4). In fact, the amount of H_2O_2 generated by the thyroid gland enzymatic system *in vitro* is similar to that produced by the glucose–glucose oxidase system used in the present tests, so it is possible that this effect could be found *in vivo* (Leseney *et al*., 1998).

Furthermore, the effect of boiled *K. brasiliensis* aqueous extract on peroxidase activity was similar to that found using non-boiled extract, suggesting that the consumption of *K. brasiliensis* as 'tea' could not avoid its effects on TPO activity *in vivo*.

However, following oral administration of drugs, a significant portion of the dose can be absorbed and metabolized (inactivated or activated) in the liver or intestines before reaching systemic circulation (Benet *et al*., 1995). But this does not seem to be the case for the TPO inhibitory activity of *K. brasiliensis* or thioureylenes, since neither *K. brasiliensis* nor thioureylenes IC_{50} were changed by treatment with hepatic microsomal enzymes (activated S9 fraction). Thus, the component of *K. brasiliensis* that causes TPO inhibition is probably not metabolized by the liver, and could reach the thyroid unchanged depending on the gastrointestinal tract absorption.

It has recently been reported that *K. brasiliensis* extracts contain some flavonoids, among them quercitrin (quercetin-3-L-rhamnoside) and isoquercitrin (quercetin-3-glucoside) (Rossi-Bergmann *et al*., 1997). On the other hand, the concentration of quercetin necessary to inhibit the TPO activity by 50% has been found to be near 2 μM, suggesting a possible antithyroid effect *in vivo* (Divi and Doerge, 1995). These results are consistent with the findings that dietary flavonoids, which are constituents of *K. brasiliensis*, could lead to decreased thyroid iodide uptake *in vivo* and could be goitrogenic (Lindsay *et al*., 1989; Moudgal *et al*., 1958). As a result, the chronic consumption of this plant extract could lead to thyroid dysfunction. There are some anecdotal references to thyroid gland increase after prolonged use of *K. brasiliensis* aqueous extract. However, this has not been confirmed since its use is mainly intermittent, and there are no studies that correlate the use of this plant extract to a high prevalence of goitre. On the other hand, there are areas of endemic goitre in the interior of Brazil, and we cannot exclude the involvement of goitrogenic substances in the pathogenesis of these goi-

tres, which are only described as being caused by iodine deficiency (Rosenthal and Ulyssea, 1978).

This is the first report on the possible antithyroid effect of *K. brasiliensis*; nevertheless, which component or components of the *K. brasiliensis* aqueous extract can be inhibitory of the *in vitro* TPO activity is yet to be determined. It has also to be established whether the *in vitro* effects are relevant *in vivo*. Considering our knowledge at this point, we believe that intermittent or low-dose exposure to *K. brasiliensis* extracts would have no significant impact on thyroid hormone status. Nevertheless, it is possible that in the near future chronic exposure could elicit prolonged blockage of thyroid hormone synthesis, if the trend for increasing use of natural products continues. As a chronic inhibition of thyroid hormone synthesis induces increased secretion of thyroid stimulating hormone, which can lead to thyroid growth, our results suggest that chronic consumption of *K. brasiliensis* has the potential to induce hypothyroidism and goitre, especially in areas with a low iodine intake.

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