

Proceedings

Xanthine Oxidase Inhibition by Aqueous Extract of *Limonium brasiliense* (Plumbaginaceae) †

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Abstract: *Limonium brasiliense* (Boiss.) Kuntze (Plumbaginaceae) is a medicinal plant, that is popularly used in the folk medicine. In this study, we examined XO inhibitory activity of the aqueous fraction of *Limonium brasiliense* extract, and its polar bioactive constituents, using the enzymatic HX–XO system *in vitro*. Seven active compounds isolated from the aqueous extract, have proved to be strong XO inhibitors. Prodelphinidin B1-3,3'-digallate (**1**), showed the most potent inhibitory activity with an IC₅₀ value of 6.61 μM, comparable to allopurinol. This study provides bases for the potential use of the aqueous fraction of *L. brasiliense* against hyperuricemia, displaying potent XO inhibitory activity with an IC₅₀ value of 48.3 μg/mL. This inhibitory effect is compatible with the presence of (**1**) in this extract.

Keywords: xanthine oxidase; Inhibition; *Limonium brasiliense*; Prodelphinidin B1-3,3'-digallate

1. Introduction

Xanthine Oxidase (XO) is enzyme that catalyzes the oxidation of hypoxanthine and xanthine to uric acid; the inhibition of xanthine oxidase decreases uric acid accumulation in the joints, controlling the disease called Gout and is relevant for the treatment of hyperuricemia. Currently, gout is treated with the drug allopurinol (hypoxanthine analog). Prolonged use of allopurinol leads to adverse effects for health, since it controls, but does not eliminate the uric acid overproduction [1]; making necessary to find new alternatives for control and gout prevention. Also, XO inhibitors are being investigated for management of reperfusion injury.

Oxidative stress, defined as an imbalance between levels of various oxidant molecules as reactive oxygen species (ROS) and antioxidants, leads to many biochemical changes and, consequently, serious disorders in the human organism. Oxidative stress can result in damage of biomolecules, such as lipids, proteins and DNA, leading to cytotoxic and genotoxic effects. Although ROS are involved in host defense, overproduction of these species may contribute to the pathogenesis of inflammatory diseases and other immunopathological phenomena related to oxidative stress, such as rheumatoid arthritis, myocardial reperfusion injury, respiratory disorders, hepatocellular oxidative DNA injury, and pathogenesis of CNS neurons [2–7].

The inhibitory activity of xanthine oxidase (XO) is a combination of uric acid formation inhibition and superoxide anion (O₂•⁻) generation suppression. The inhibition of uric acid formation by XO is useful for the screening of natural compounds that prevent gout, while the suppression of O₂•⁻ generation is useful for treating oxidative stress. The plants contain abundant phenolic compounds and some of these compounds, flavonoid like have shown to inhibit XO and reactive oxygen species [8]. In recent studies, it has been demonstrated that XO inhibitory activity and the suppression of O₂•⁻ generation, are used to characterize natural products [9].

Limonium brasiliense (Boiss.) Kuntze (Plumbaginaceae) is a medicinal plant known by its common name “guaycurú”, that grows mostly in saline soils, distributed in Argentina, Uruguay and South of Brazil. Infusion from the roots is popularly used in the treatment of hemorrhage, menstrual disorders, rheumatism, and it is believed to have cardioprotective properties [10].

In previous studies we have evaluated the methanolic extract from the roots of *L. brasiliense* that showed antioxidant activity evidenced by the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) [11]. For this reason, we examined XO inhibitory activities of the aqueous fraction of *L. brasiliense* extract, and its polar bioactive constituents, using the enzymatic HX–XO system *in vitro*. Our results suggest that the antioxidant activity of the aqueous fraction of *L. brasiliense* extract may be partially explained by an $O_2^{\bullet-}$ scavenging effect and by inhibition of XO activity.

For the first time, this study provides a new potential alternative for the use of *L. brasiliense* against hyperuricemia and for control and gout prevention.

2. Material and Methods

2.1. General

1H and ^{13}C NMR spectra were recorded in DMSO- d_6 using TMS as internal standard on a Bruker ARX 300 multinuclear spectrometer at 300 MHz and 75 MHz, respectively. UV spectra were recorded on a GBC Spectral UV-VIS spectrophotometer.

Silica gel 60 (70–230 mesh, Fluka) was used for column chromatography, DCVC chromatography with C18 silica and semipreparative HPLC with a C₁₈ Phenomenex Gemini column.

UV spectra were recorded on a GBC Spectral UV-VIS spectrophotometer and microplate UV/VIS reader (Titertek Multiscan, Flow Laboratories, Finland). Fluorescence emission was then determined in a microplate fluorescence reader (Bio-Tek Instruments, UK; excitation 488 nm, detection 530 nm) over a 55 min period and microplate ELISA reader (Fluo-star Optima, BMG LABTECH, Offenburg, Germany)

Nitroblue tetrazolium (NBT), hypoxanthine (HX), xanthine, superoxide dismutase (SOD; from bovine erythrocytes), phosphate buffer (KH₂PO₄-KOH), EDTA-Na₂, allopurinol and XO enzyme (from buttermilk) were all purchased from Sigma. Appropriate dilutions of the above drugs were prepared every day immediately before use in PBS (XO assays), and/or in deionized water (for the other experiments) from the following concentrated stock solutions kept at 20 °C: SOD (1 U/mL) in deionized water, HX and xanthine (10 mM) in a 0.1% *w/v* aqueous KOH solution; all other compounds (100 mM) in deionized water. XO was dissolved daily before the experiments in PBS.

2.2. Plant Material

Roots of *L. brasiliense* were collected at Puerto Cuatros (38°45' S, 62°22' O), General Cerri, Buenos Aires province, Argentina, in February 2010. A voucher specimen was identified by Dr. María Gabriela Murray and was deposited in the Herbarium of Universidad Nacional del Sur (BBB) in Bahía Blanca, Argentina, under the number Murray, M.G. 421.

2.3. Obtention of Aqueous Extract and Its Active Compounds

Dried roots (124.3 g) were macerated overnight with ethanol (EtOH) (1 L). The ethanolic extract (EE) was evaporated *in vacuo* to yield 78.2 g of a brown residue that was dissolved in water (200 mL) and partitioned with hexane (HF, 3 × 150 mL). The aqueous fraction (AF) was evaporated and the residue was lyophilized for 3 days (45.04 g). A portion of AF (0.7 g) was submitted to DCVC chromatography with C18 silica and H₂O:MeOH as eluent (100:0, 90:10, 80:20, 70:30, 50:50, 30:70, 0:100). This procedure was repeated 5 times. Chromatographic separation was monitored by TLC and the fractions were pooled according to their chromatographic profile in seven fractions F1-F7. Fractions F2, F3 and F4 were selected for further chromatographic separation due to their activity in the DPPH assay. These fractions were submitted to semipreparative HPLC with a C₁₈ Phenomenex Gemini column (5 μm C₁₈ 110 Å, 250 × 10 mm), and MeOH:H₂O (50:50) as mobile phase (1 mL/min). Absorbance was registered at λ = 254 nm. Compound 7 (8.2 mg) was isolated from the F2/A fraction

and compound **1** (31.9 mg) from the F2/C fraction, with a retention time of 3.1 and 11.2 min, respectively. Compound **6** (20.3 mg) was isolated from the F3/A fraction and compound **2** (29.4 mg) from the F/B fraction, with a retention time of 8.7 and 10.8 min, respectively.

Compound **3** (61.4 mg) was obtained from F3/C and F4/C fractions, with a retention time of 25.2 min. Compounds **4** (30.5 mg) and **5** (6.1 mg) were obtained from F4/A and F4/B fractions, with a retention time of 16.5 and 18.3 min, respectively. All compounds were characterized by ^1H and ^{13}C RMN.

2.4. Xanthine Oxidase Activity

Generation of $\text{O}_2^{\bullet-}$ by use of the enzymatic hypoxanthine oxidase the HX-XO system.

The superoxide ion ($\text{O}_2^{\bullet-}$) was generated enzymatically in an HX-XO system and quantified by the spectrophotometric measurement of the product of the reduction of NBT, essentially following the procedure described by Orallo et al. [12]. Briefly, 250 μL of test solution of 1mM EDTA- Na_2 , 100 μM HX, 100 μM NBT in phosphate buffer (50 mM KH_2PO_4 -KOH, pH 7.4) and EE or AF extracts (0.1–250 $\mu\text{g}/\text{mL}$) and compounds **1–7** (0.1–500 μM). Control experiments were carried out simultaneously using the same test solution but without extract. The reaction was started with the test solution already in an Cobas Fara 22–3123 AutoAnalyzer (Roche), by adding XO in phosphate buffer (0.066 U/mL) and continued at room temperature (22–24 $^\circ\text{C}$) for 10 min. The rate of NBT reduction was estimated from the difference in absorbance at 560 nm between the test solution and a blank solution in which the XO was replaced by buffer solution. In some experiments, the sensitivity of the method was evaluated by assessing the influence of SOD (1 U/mL), a known scavenger of $\text{O}_2^{\bullet-}$.

Determination of XO activity by use of the xanthine- XO system.

The potential effect of *L. brasiliense* extracts and compounds **1–7** on XO activity was investigated by measuring uric acid formation, as previously described (Orallo et al., 2002). Briefly, 1 mL of test solutions of phosphate buffer (50 mM KH_2PO_4 -KOH, pH 7.4) containing the EE or AF extract at different concentrations (0.1–250 $\mu\text{g}/\text{mL}$) and compounds **1–7** at different concentrations (0.1–500 μM), EDTA- Na_2 (1 mM), and 0.066 U of XO were incubated for 15 min at room temperature. The reaction was started by adding xanthine in phosphate buffer (100 μM), and the rate of uric acid production was estimated from the difference in absorbance at 295 nm (measured at room temperature for 10 min in a UV-visible absorption spectrophotometer, Shimadzu UV-240, Duisburg, Germany) between the test solution and a blank solution in which XO was replaced by buffer solution. In some assays, the validity of the method was confirmed by assessing the influence of allopurinol (a well-known XO inhibitor) on uric acid formation.

2.5. Antioxidant Activity

Determination of DPPH assay

The antioxidant activity was evaluated through the ability as free radical scavenger of extracts, fractions and/or pure compounds, as described Bors et al. [13]. The preliminary test was performed with a rapid TLC screening method using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Analytical TLC on reverse plates was developed with appropriate conditions after application of 5 μL of each test compound solution (1 mg/mL), dried and sprayed with DPPH solution (0.2%, MeOH). 5 min later active compounds appeared as yellow spots against a purple background. The purple stable free radical 2,2-diphenyl-1-picrylhydrazyl was reduced to the yellow colored diphenylpicryl hydrazine. Quercetin was used as positive control.

2.6. Statistical Analysis

Statistical analysis was performed using Tukey–Kramer correction of a one-way ANOVA test using Graph-Pad Prism 5.0 software. The results are expressed as means standard errors of the means (SEM). The concentration of drugs that caused a 50% inhibition of the response (IC_{50}) was estimated by nonlinear regression analysis (GraphPad Prism software; GraphPad, San Diego, CA, USA) from the concentration-response curves obtained in each case

3. Results and Discussion

3.1. Extraction and Isolation

Dried roots of *L. brasiliense* were milled and macerated with ethanol to obtain ethanolic extract (EE) that was dissolved in water (AF) and partitioned with hexane (HF). AF was submitted to chromatographic separations to render seven pure compounds that were identified as Prodelphinidin B1-3,3'-digallate (1), myricetin (2), apigenin (3), taxifolin (4), 3-O-acetyltaxifolin (5), myricetin-3-O- α -rhamnopyranoside (6) and gallic acid (7). Identification was achieved on the basis of their ^1H and ^{13}C NMR spectra and by comparison with literature data [14–16]

3.2. Xanthine Oxidase Activity

XO catalyzes the formation of uric acid and ROS during the catabolism of purines, and excess of uric acid can lead to increased oxidative stress, mutagenesis and possibly, to cancer development [17]. In recent studies, it has been demonstrated that XO inhibitory activity and the suppression of $\text{O}_2^{\bullet-}$ generation, are used to characterize natural products. It has been reported that XO converts HX or xanthine to uric acid, H_2O_2 , and $\text{O}_2^{\bullet-}$ [18]. $\text{O}_2^{\bullet-}$ generated by this system react with NBT to produce formazan (NBT is reduced in the reaction). The formation of this colored compound (formazan) and the amount of $\text{O}_2^{\bullet-}$ generated enzymatically, may be measured spectrophotometrically [19]. When a drug lowers the amount of $\text{O}_2^{\bullet-}$ (i.e., the reduction of NBT) and at the same time does not affect the formation of uric acid, it is considered to be a selective scavenger of $\text{O}_2^{\bullet-}$. On the other hand, if a drug inhibits XO activity, both uric acid and $\text{O}_2^{\bullet-}$ concentrations are diminished.

XO is a key enzyme which can catalyze hypoxanthine and xanthine to uric acid causing hyperuricemia in humans. In this study, we examined XO inhibitory activities of aqueous fraction of *L. brasiliense* extract and its polar bioactive constituents, using the enzymatic HX–XO system *in vitro*, which can be measured spectrophotometrically.

Superoxide-scavenging activity of the aqueous fraction and isolated compounds of *L. brasiliense*

To investigate the possible superoxide-scavenging activity, we performed assays in which $\text{O}_2^{\bullet-}$ was generated by an enzymatic system (HX–XO). EE and AF at concentrations between 0.1 and 250 $\mu\text{g}/\text{mL}$ significantly reduced medium concentrations of $\text{O}_2^{\bullet-}$, with the inhibitory effect with IC_{50} values of $42.03 \pm 1.04 \mu\text{g}/\text{mL}$ and $10.86 \pm 1.25 \mu\text{g}/\text{mL}$, respectively. (Figure 1).

In this assay myricetin (2) and myricetin-3-O- α -rhamnopyranoside (6) showed a significant superoxide-scavenging activity with IC_{50} values of $6.04 \pm 1.51 \mu\text{M}$ and $18.96 \pm 1.36 \mu\text{M}$, respectively. A moderate effect was observed for taxifolin (4) with an $\text{IC}_{50} = 35.93 \pm 1.65 \mu\text{M}$. Neither apigenin (3) ($\text{IC}_{50} 67.21 \pm 1.35 \mu\text{M}$) nor gallic acid (7) ($\text{IC}_{50} 126.13 \pm 2.06 \mu\text{M}$) had significant effects.

Prodelphinidin B1-3,3'-digallate (1), showed the most potent superoxide-scavenging activity with an IC_{50} value of $1.58 \mu\text{M}$ (Figure 2).

The results obtained for each sample are summarized in Figures 1 and 2 and Table 1.

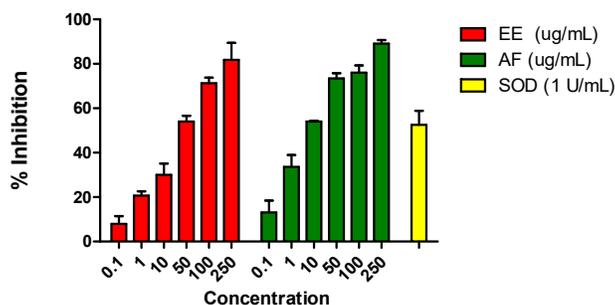


Figure 1. Effects of the ethanolic extract (EE) and aqueous fraction (AF) (0.1–250 $\mu\text{g}/\text{mL}$) of *L. brasiliense* on reduction of NBT by $\text{O}_2^{\bullet-}$ generated by XO in the presence of 100 μM HX. The rightmost bar represents assays in which the enzyme superoxide dismutase (SOD; 1 U/mL) was added to

cultures instead of EE and AF; this enzyme abolishes superoxide generation in the HX-XO system. Values shown are mean percentages of the control response ($n = 3$) \pm SEM. Asterisks indicate statistical significance ($p < 0.001$).

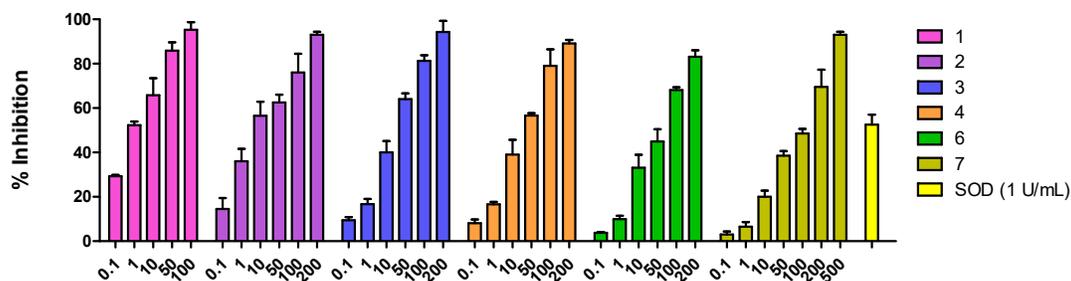


Figure 2. Effects of the compounds 1–7 (0.1–500 μ M) on reduction of NBT by $O_2^{\bullet-}$ generated by XO in the presence of 100 μ M HX. The rightmost bar represents assays in which the enzyme superoxide dismutase (SOD; 1 U/mL) was added to cultures instead of EE and AF; this enzyme abolishes superoxide generation in the HX-XO system. Values shown are mean percentages of the control response ($n = 3$) \pm SEM. Asterisks indicate statistical significance ($p < 0.001$).

Table 1. IC_{50} values SOD and XO of extracts and isolated compounds obtained from *L. brasiliense*.

Antioxidants	IC_{50} SOD ^a	IC_{50} XO ^a
allopurinol ^b	N.T	3.61 ± 0.05 ^c
EE	42.03 ± 1.12 ^d	96.14 ± 1.12 ^d
AF	10.86 ± 1.53 ^d	48.3 ± 1.53 ^d
prodelphinidin B1-3,3'-digallate (1)	1.85 ± 0.13 ^c	6.61 ± 0.13 ^c
myricetin (2)	6.04 ± 0.25 ^c	6.89 ± 0.25 ^c
apigenin (3)	67.21 ± 0.22 ^c	7.01 ± 0.22 ^c
taxifolin (4)	35.93 ± 0.36 ^c	31.58 ± 0.36 ^c
myricetin-3-O- α -rhamnopyranoside (6)	18.96 ± 1.02 ^c	167.02 ± 1.02 ^c
gallic acid (7)	126.13 ± 1.35 ^c	213.24 ± 1.35 ^c

^a Values are the mean \pm S.E.M. of three replicas. ^b Positive control ^c μ M. ^d μ g/mL. NT not tested.

Effects of the aqueous fraction and compounds 1–7 on XO activity

The effects of aqueous fraction of *L. brasiliense* extract and its polar bioactive constituents (1–7) on XO activity were investigated by measuring their ability to affect the formation of uric acid from xanthine.

Only AF showed inhibition of uric acid formation, and thus XO activity with an IC_{50} value of 48.30 ± 1.63 μ g/mL, while EE showed a moderate inhibition of uric acid formation ($IC_{50} = 96.14 \pm 2.09$ μ g/mL) (Figure 3).

As is shown in Figure 4, neither myricetin-3-O- α -rhamnopyranoside (6) (IC_{50} 167.02 ± 1.22 μ M) nor gallic acid (7) (IC_{50} 213.24 ± 2.06 μ M) had significant effects, while taxifolin (4) elicited a moderate inhibition of uric acid formation ($IC_{50} = 31.58 \pm 1.78$ μ g/mL). On the other hand, myricetin (2) and apigenin (3), showed a significant inhibition of XO activity with IC_{50} values of 16.89 ± 1.03 μ M and 19.01 ± 1.10 μ M, respectively.

Prodelphinidin B1-3,3'-digallate (1), showed the most potent inhibitory activity with an IC_{50} value of 6.61 μ M, comparable to allopurinol, the drug of choice for inhibition of XO in gout patients.

The results obtained for each sample are summarized in Figures 3 and 4 and Table 1.

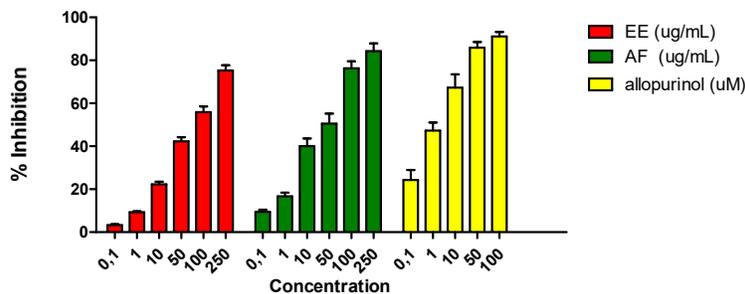


Figure 3. Effects of the ethanolic extract (EE) and aqueous fraction (AF) (0.1–250 $\mu\text{g/mL}$) of *L. brasiliense* on uric acid production by XO in the presence of 100 μM xanthine. The rightmost bar represents assays in which the XO inhibitor allopurinol was added. Values shown are means percentages of the control response ($n = 3$) \pm SEM. Asterisks indicate statistical significance ($p < 0.001$).

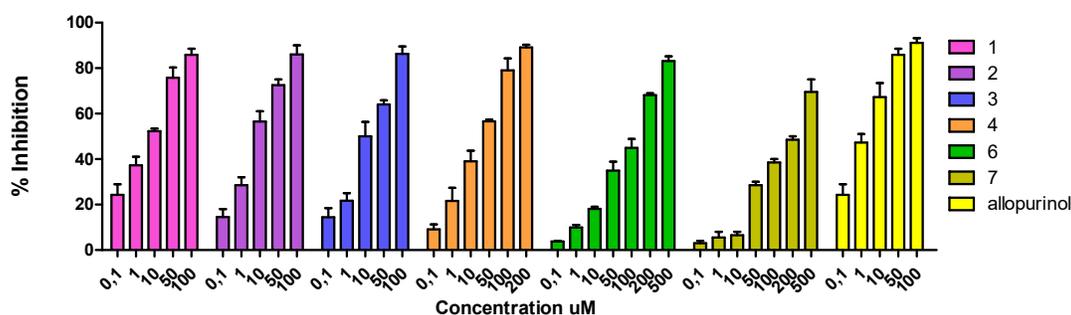


Figure 4. Effects of the compounds 1–7 (0.1–500 μM) of *L. brasiliense* on uric acid production by XO in the presence of 100 μM xanthine. The rightmost bar represents assays in which the XO inhibitor allopurinol was added. Values shown are means percentages of the control response ($n = 3$) \pm SEM. Asterisks indicate statistical significance ($p < 0.001$).

4. Conclusions

In the present study, the effects of the aqueous fraction of *L. brasiliense* extract, and its polar bioactive constituents on XO activity were investigated by measuring their ability to affect the formation of uric acid from xanthine, using the enzymatic HX–XO system in vitro and oxidative stress inhibition using $\text{O}_2^{\bullet-}$ scavenging effect.

The aqueous fraction of *L. brasiliense* displayed potent XO inhibitory activity with an IC_{50} value of 48.3 $\mu\text{g/mL}$. Our result suggests that the anti-oxidant activity of the aqueous fraction of *L. brasiliense* extract previously reported [11] may be partially explained by an $\text{O}_2^{\bullet-}$ scavenging effect (IC_{50} 10.86 \pm 1.25 $\mu\text{g/mL}$) and by inhibition of xanthine oxidase (XO) activity.

This inhibitory effect is compatible with the presence of flavonoid compounds in this aqueous fraction. It has been previously demonstrated that myricetin (2) and apigenin (3) can inhibit XO activity [20,21].

Prodelphinidin B1-3,3'-digallate (1) was effective for scavenging superoxide radicals ($\text{O}_2^{\bullet-}$) (IC_{50} 1.85 \pm 0.13 μM), generated enzymatically by a hypoxanthine (HX)/xanthine oxidase (XO) system and for inhibiting XO activity. This compound showed the most potent XO inhibitory activity with an IC_{50} value of 6,61 μM , at the same level as allopurinol, the drug of choice for inhibition of XO in gout patients. These results clearly indicate that 1 is display selective $\text{O}_2^{\bullet-}$ - scavenging and direct inhibitory XO properties

In conclusion, the aqueous fraction of *L. brasiliense* extract significantly inhibits ROS production, both by inhibiting XO and by scavenging ROS already produced. It seems likely that these activities are attributable to Prodelphinidin B1-3,3'-digallate (1) present in the extract.

For the first time, this study provides a new alternative for the use of aqueous fraction of *L. brasiliense* against hyperuricemia and for control and gout prevention. Thus, flavonoids could be used as a preventive and therapeutic agent for gout. In addition, they might be useful as a biochemical probe, taking advantage of their potent inhibitory activities.

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