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Anti-inflammatory, Antioxidant and Antimicrobial Activities of Extracts from Ecolite® Waist Tonic

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ABSTRACT

Waist tonics are commonly used as a traditional remedy for pain relief, energy enhancement, and overall health improvement. Ecolite® Waist Tonic (EWT) extract contains the traditional herbal ingredients used in waist tonic. With reported over 500,000 cases of low back pain worldwide in 2020, with a projection of 843 million by 2050, the demand for effective medication is urgent. The use of COX-2 inhibitors is essential in managing inflammation, particularly in conditions like back pain. Therefore, this study sought to evaluate the *in vitro* COX-2 inhibitory activity together with antioxidant and antibacterial activities of EWT extract to understand the scientific basis for its traditional use in managing pain and inflammation. COX-2 inhibitory activity exhibited EWT extract was evaluated using an enzyme-based assay. Additionally, FRAP, DPPH and ABTS assays were used to assess the antioxidant activity of EWT extract, whereas antimicrobial activity was tested against two pathogenic bacteria and two fungi. Although the moderate antioxidant activity and weak antimicrobial properties exhibited by EWT extract, a strong COX-2 inhibition of 72% was found in EWT extract, which is comparable with

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77% inhibition of celecoxib as a positive control. These results support its potential use in managing and controlling inflammation.

Keywords: Antioxidant activity, antimicrobial activity, COX-2 inhibition, Ecolite® waist tonic, traditional Chinese medicine

INTRODUCTION

Low back pain (LBP) is ranked in the top ten most frequently attended illnesses by the primary healthcare professionals (Kuritzky & Samraj, 2012). About 70% of the world population complained of suffering acute or chronic back pain throughout their lifetime (Strine & Hootman), which resulted in 619 million cases of LBP reported worldwide in 2020. In a projection towards 2050, there will be an estimated 843 million people worldwide who will suffer LBP (Ferreira et al., 2023). In Malaysia, about 12% of the total population, or 3.8 million Malaysians, are reported to acquire LBP (Sivasampu et al., 2014). There is a significantly higher rate of LBP cases found among the high-risk groups. The prevalence of LBP has been reported to affect Malaysian, ranging from 12.4% to 84.6% across different groups of residents (Daud et al., 2023). It is expected to rise over the years, as a result of the changes in lifestyle after the COVID-19 pandemic, such as work-from-home policies and post-pandemic social practice alterations (Muniandy et al., 2022). The elevating trend of LBP cases resulted in societal and economic burden, particularly in the low- and middle-income countries, as it causes increasing direct and indirect healthcare costs. In addition, the impact also propagated to the incremental early retirement personnel due to the frequent absenteeism at work and the depletion in work productivity (Hartvigsen et al., 2018). It is therefore a dire need to seek solutions in managing and preventing LBP.

Oxidative stress, microbial infections, and inflammation can be classified as the main causes of many health complications. Antioxidants are known to neutralise free radicals that are associated with chronic diseases (Lobo et al., 2010), while antimicrobial agents can help to combat infections. Cyclooxygenase (COX)-2 inhibitors are crucial in managing inflammation, making them useful in treating conditions such as arthritis and back pain (Fine, 2002). The treatment of LBP with COX-2 inhibitors is well-established, but can be a complicated topic in pain management. COX-2 inhibitors (the coxibs series) are a subclass of nonsteroidal anti-inflammatory drugs (NSAIDs) that were developed to offer the pain-relieving benefits of traditional NSAIDs (van der Gaag et al., 2020). Although the use of coxibs series has a reduced risk of gastrointestinal (GI) side effects, there are reports of increased risk of cardiovascular events, including heart attack and stroke, which led to the withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra) from the market (Bennett et al., 2005).

Ecolite® Waist Tonic (EWT) is a natural herbal formulation crafted with a combination of traditional Chinese herbs including *Eucommia ulmoides* (du zhong), *Achyranthes bidentata* (Niu xi), *Panax ginseng* (ren shen), *Cordyceps sinensis* (dong chong xia cao), *Ganoderma lucidum* (ling zhi), *Psoralea corylifolia* (buguzhi) and *Lycium Barbarum* (Gouqizi), in combination with other 10 ingredients, namely *Ligusticum chuanxiong*, *Polygonatum sibiricum*, Morinda officinalis, *Rehmannia glutinosa*, *Salvia miltiorrhiza*, *Citrus reticulata*, *Schisandra chinensis*, *Sophora flavescens*, *Citrus aurantium*, and *Glycyrrhiza uralensis.* A standardised tongkat ali extract *(Eurycoma longifolia)* is also added to this remedy to elevate the overall functional properties as a traditional local herb. (NPRA, 2025). EWT is made to alleviate lower back pain, boost energy and vitality, combat fatigue, enhance blood circulation and support overall health.

In the effort of seeking a potential supportive LBP management alternative, in this study, we focused on the evaluation of COX-2 inhibitory activities in complement with antioxidant and antimicrobial activities of EWT extract via *in vitro* assays, offering insights into its potential applications in modern healthcare.

MATERIALS AND METHODS

Materials

EWT was provided by Ecolite Biotech Manufacturing Inc. as blended plant extracts in aqueous form (4% w/v). The extracts were freeze-dried prior to further analysis. Chemicals used for this work include Elabscience COX-2 Inhibitor Screening kits (E-BC-D002) and four positive controls or reference drugs (celecoxib, quercetin, nystatin and streptomycin sulfate), ferrous sulfate ($FeSO_4$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 3-ethylbenzo-thiazoline-6- sulfonic acid (ABTS), dimethyl sulfoxide (DMSO), Pathogenic bacterial and fungal strains used for antimicrobial tests were including *Bacillus subtilis*, *Streptococcus pyogenes*, *Candida albicans* and *Aspergillus niger*.

Experimental Setup

COX-2 Inhibition Assay

This assay was conducted according to the test protocols provided by the Elabscience COX-2 inhibition assay kit (E-BC-D002). The assay also utilised a water bath, an analytical balance, VANTAstar multi-mode microplate reader (BMG LABTECH), micropipette and microcentrifuge tubes. EWT solution (400 mg/mL) was prepared by dissolving 400 mg Ecolite extract in 1 mL dimethyl sulfoxide (DMSO). Working concentrations were prepared by subsequent dilution in DMSO to yield final concentrations of 200 mg/mL and 100 mg/mL, respectively. The accelerator, chromogen and substrate solution were mixed thoroughly

and frozen before use of the kit. The reagents were protected from light for the whole experiment. Celecoxib (purity >98%, 38 µg/mL) was used to replace the tested sample solution as a positive control. The blank containing only the assay buffer was employed as the background reference for the measurement of fluorescence. The negative control group was the group containing recombinant COX-2 enzyme without any inhibitor and was set to be the maximum or 100% initial enzyme activity. The experiment was performed on a black 96-well microplate. Initially, 140 µL of assay buffer was pipetted into each well prepared for assay. 10 µL of the test sample solution and 10 µL of the positive control solution of celecoxib were added to the respective wells. 10 µL assay buffer was added to the negative control wells in place of the inhibitor solution. Subsequently, 20 µL of recombinant COX-2 enzyme solution was added to all wells except blank wells. The reaction mixture was then incubated at room temperature for 5 min to enhance the interaction of the inhibitor with the enzyme. After incubation, 10 µL of the chromogenic agent and 10 µL of the accelerator were added to each well. Later, 10 µL of substrate solution was added to each well to activate the enzymatic reaction. The plate was gently shaken and incubated in the dark at 37 °C for 30 minutes. The fluorescence intensity was then immediately measured with a fluorescence microplate reader (excitation and emission wavelengths were 535 nm and 587 nm, respectively). The inhibition percentages were calculated by comparing the absorbance values of the tonic extracts to those of untreated control enzymes.

Antioxidant Activity

The antioxidant activity of the waist tonic extracts was evaluated using three complementary assays, i.e., ferric reducing antioxidant power (FRAP) assay, DPPH radical scavenging assay and ABTS assay. Quercetin (≥95% purity via HPLC) was used as a positive control in all three assays.

FRAP Assay

The ferric reducing antioxidant power (FRAP) assay was performed following the previous method with modifications (Benzie & Strain, 1996). 10 µL of methanolic extract at two various concentrations (1 and 10 mg/mL; three replicates per sample and concentration) was mixed with 300 µL of freshly prepared FRAP reagent. The FRAP reagent was prepared from 10 parts of 300 mM sodium acetate buffer (pH 3.6), 1 part of 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), and 1 part of 20 mM $FeCl_3$ hexahydrate. The mixture was incubated at 37 °C for about 4 minutes. A microplate reader (Versamax™) is used to measure the sample absorbance at 470 nm. A blank control was prepared by replacing the methanolic extract with methanol. The results were expressed as milligram equivalents of $FeSO_4$ per milligram of dry extract weight. The assay was carried out in triplicate.

DPPH Radical Scavenging Assay

The DPPH assay was carried out using a published method with slight modifications (Güllüce et al., 2003). Ten milligrams (mg) of EWT extract was dissolved in 1 mL of methanol. This stock solution was then further diluted with methanol to ten different concentrations (0.0195, 0.0391, 0.0781, 0.1563, 0.3125, 0.6250, 1.2500, 2.5000, 5.0000 and 10.0000 mg/mL). The samples were then centrifuged at 10000 rpm for three minutes at 4 °C (Eppendorf 5415R centrifuge, Hamburg, Germany). 100 µl of each diluted sample was mixed with 100 µl of a 0.1 mg/mL DPPH solution in methanol in the 96-well plates and incubated at room temperature for 30 minutes. Their absorbance was subsequently obtained at 517 nm against a blank using a VersaMaxTM microplate reader. The percentage of DPPH radical inhibition (I%) was calculated using Equation 1:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100 \quad [1]$$

Where A_{blank} is the absorbance of the control reaction that contains all reagents except the test compound, and A_{sample} is the absorbance of the test compound. The 50% inhibition (IC_{50}) of the extract was determined by plotting the percentage inhibition against the extract concentrations and was calculated from the resulting curve. The assay was carried out in triplicate.

ABTS Assay

The antioxidant activity of the extracts against $ABTS^{+}\bullet$ radicals was carried out using the method that has been published previously (Stratil et al., 2006). The $ABTS^{+}\bullet$ radical cation was initiated by mixing 7 mM $ABTS^{+}\bullet$ with 4.95 mM potassium persulfate in a 1:1 (v/v) ratio. This mixture was then kept in the dark for 12 hours at room temperature. The resulting solution was then diluted with methanol until its absorbance hit between 0.6 and 0.7 at 734 nm wavelength. Ten milligrams (mg) of the samples were dissolved in 1 mL of methanol. This stock solution was then further diluted with methanol to ten different concentrations (0.0195, 0.0391, 0.0781, 0.1563, 0.3125, 0.6250, 1.2500, 2.5000, 5.0000 and 10.0000 mg/mL). 10 µl of these diluted samples was transferred into a 96-well plate and added to 300 µl of ABTS+● solution. The plate was incubated for 10 minutes at room temperature, and the absorbance was read at 743 nm wavelength using a VersaMax™ microplate reader. Methanol was used as a blank. A blank control was prepared using $ABTS^{+}\bullet$ solution, and results were expressed as percent inhibition. Quercetin was used as a positive control by experimenting with its series of concentrations ranging from 0.0020 mg/mL to 1 mg/mL. The assay was carried out in triplicate.

Antimicrobial Activity

Disk Diffusion Assay

The freeze-dried EWT extracts were dissolved in DMSO to a final concentration of 400 mg/mL and filtered through 0.45 µm Millipore filters. Antimicrobial activity was assessed using the disk diffusion method (Güllüce et al., 2003). A suspension of 10^8 CFU/mL of bacteria or 10^6 CFU/mL of fungi was spread onto nutrient agar (NA) or potato dextrose agar (PDA) media, respectively. Disks (6 mm in diameter) were impregnated with 400 mg/mL of the EWT extracts prepared earlier and placed on the inoculated agar. Negative controls were prepared using the solvent blank, i.e. DMSO. Streptomycin sulfate (1 mg/mL) was used as a positive control for bacterial strains, whereas nystatin (100 IU/disk) was used as a positive control for fungal strains. In the case of the antibacterial study, the inoculated plates were incubated for 24 hours at 37 °C, whereas in the case of the antifungal study, the inoculated plates were incubated for 48 hours at 30 °C. These antimicrobial strengths were evaluated by measuring the diameter of their resulting inhibition zones around the disks and were compared with the negative and positive controls. Each assay was carried out in triplicate.

Statistical Analysis

Data was analysed using the Statistical Package for Social Sciences (SPSS) (version 30.0) software. Statistical analysis was conducted using one-way ANOVA (analysis of variance) to compare the means of different groups. All experiments were carried out in triplicate, and the results are expressed as the mean ± standard deviation (SD). In the case of the COX-2 inhibition assay, a one-way ANOVA was performed to compare the mean of each EWT concentration column with the mean of the control column, followed by Dunnett's multiple comparison tests to determine their statistical significance, where they were denoted as $p < 0.05$, $p < 0.001$, and $^{**}p < 0.0001$.

Normality Testing and QQ Plot Analysis

All data of COX-2 inhibitory activity were tested for normality with the GraphPad Prism 9.3.1 software package before the statistical analysis. Percentage inhibition data were calculated from triplicate experiments (n = 3) for positive control and EWT samples (100, 200 and 400 mg/mL) and were entered into GraphPad Prism. The “Column statistics” and “Normality and Lognormality Tests” in GraphPad Prism were used to examine the data for normality. The software produced a Normal Quantile-Quantile (QQ) plot, which compared the observed data distribution against the expected theoretical normal distribution. The QQ plot was used to plot the actual observed values against the predicted normal values, and normalcy was assessed by how close the data points were to the reference diagonal line.

RESULTS AND DISCUSSION

COX-2 Inhibitory Activity

As demonstrated in Table 1 and Figure 1, the EWT extract was found to exhibit COX-2 inhibitory activity in a concentration-dependent manner. In addition, the standard QQ plot shown in Figure 2 indicates that most data points of the EWT extract groups and positive control groups converged near the reference diagonal line. These analyses imply that data were generally distributed in a normal manner and were suited for parametric statistical analysis with one-way ANOVA followed by Dunnett's multiple comparison tests.

Table 1
Percentage of COX-2 inhibition by Ecolite® Waist Tonic (EWT)

Sample	Percent Inhibition (%)
Celecoxib (Positive Control)	76.73 ± 0.86
400 mg/mL EWT	72.42 ± 2.43*
200 mg/mL EWT	70.53 ± 1.10**
100 mg/mL EWT	65.91 ± 1.76***

Note. Values are expressed as Mean ± SD (n=3). *p<0.05, **p<0.001, and ***p<0.0001, indicating they are statistically significant. One-way ANOVA (analysis of variance) was performed to compare the mean of each EWT concentration with the mean of the positive control column, followed by Dunnett's multiple comparison tests

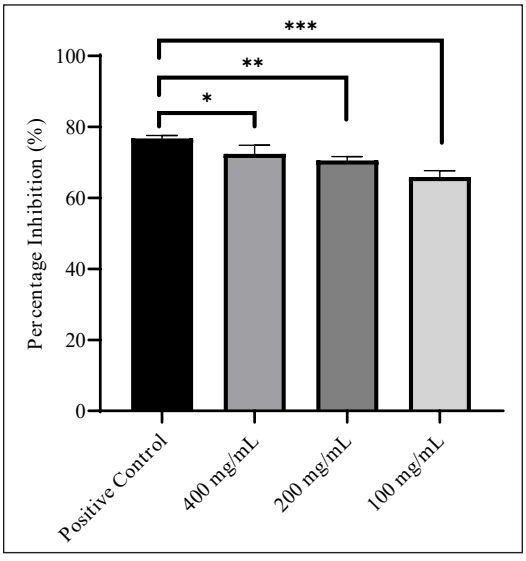

Figure 1. Bar chart representation for the percentage inhibition of COX-2 by different concentrations of EWT extract (400 mg/mL, 200 mg/mL, and 100 mg/mL) in comparison with celecoxib as a positive control. Error bars were indicated to the propagated standard errors of the mean. Data are expressed as Mean ± SD (n = 3) (*p<0.05, **p<0.001, and ***p<0.0001 versus positive control), while one-way ANOVA (analysis of variance) was performed, followed by Dunnett's multiple comparison tests, indicating that they are statistically significant

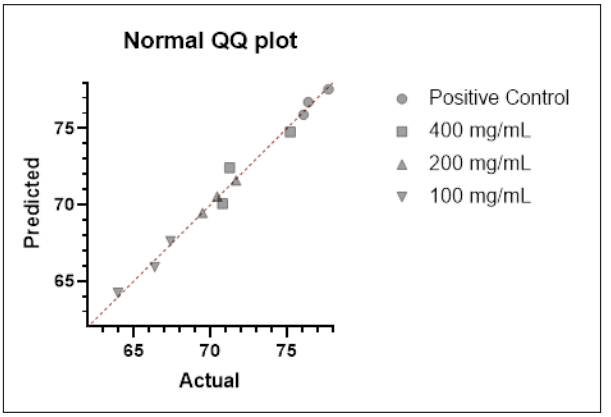

Figure 2. Normality and lognormality test (Normal QQ plot) of different concentrations of EWT extract (400 mg/mL, 200 mg/mL, and 100 mg/mL) tested against the COX-2 inhibition assay

At a concentration of 400 mg/mL, the EWT extract was found to exhibit a substantial inhibitory activity of 72.42%, which is comparable to that of celecoxib with 76.73% inhibition (Table 1). At 100 mg/mL of EWT extract that exhibited 65.91% of COX-2 inhibition, the IC_{50} of EWT extract can be approximated to be lower than 100 mg/mL. Both standard and EWT extracts exhibited apparent anti-inflammatory effects, where the performance exhibited by EWT highlights its potential as one of the natural alternatives. While celecoxib appears as a single compound with high potency, the observed slight difference in terms of inhibitory efficacy could be attributed to the complex composition of EWT that contains a blend of various strength bioactive compounds. However, the multifaceted nature of the extract could somehow provide additional therapeutic benefits, such as synergistic effects and or, reduced side effects, making it a promising candidate for further investigation. Similarly, at concentrations of 200 mg/mL and 100 mg/mL, the extract remained to exhibit notable inhibitory activity, with mean inhibition values of 70.53% and 65.91%, respectively. While these high concentrations demonstrate *in vitro* potential, they may not necessarily reflect *in vivo* bioavailability.

These findings suggest that EWT extract may contain bioactive compounds that are capable of inhibiting COX-2 activity, which appears to agree with its traditional use as an anti-inflammatory remedy. In addition, it aligns with the broader understanding of plant extracts that often contain bioactive compounds that can modulate inflammatory pathways. Knowing COX-2 is a key enzyme in the production of prostaglandins that mediates inflammation (Termer et al., 2021), the complex composition of plant extracts, including flavonoids and other secondary metabolites, can provide a multifunctional approach in reducing inflammation by disrupting various pathways involved in the inflammatory response (Attiq et al., 2018). This multi-targeted action may offer advantages over

single-compound drugs like celecoxib, by potentially reducing side effects and enhancing therapeutic efficacy via synergistic interactions exhibited by the bioactive compounds present in the extract.

Antioxidant Activity

In the FRAP assay, the reducing potential of the EWT extract was evaluated at varying concentrations, where a higher FRAP value corresponds to stronger antioxidant activity. For the DPPH and ABTS assays, the IC_{50} value representing the concentration of the sample required to inhibit 50% of free radical activity was determined, with a lower IC_{50} value indicating greater antioxidant potency (Irawan et al.,2022). Quercetin was used as a positive control, whereas methanol was used as a negative control in the FRAP assay. In the DPPH and ABTS assays, methanol is utilised as a vehicle control. It was analysed to rule out its effect towards radical scavenging activity. Since the solvent did not exhibit significant inhibition, the IC_{50} values of methanol were classified as 'Not Applicable' under DPPH and ABTS assays.

Generally, Table 2 demonstrated a moderate level of antioxidant activity of the EWT extract. In comparison to quercetin (positive control), the antioxidant potency of EWT extract was relatively lower. This observation could be attributed to the composition of the EWT that appears as a mixture of compounds, which are likely to contain a lower concentration of the specific compound(s) responsible for antioxidant activity (Spiegel et al., 2020). In the FRAP analysis, this trend was particularly apparent, where the $FeSO_4$ equivalent increased 11-fold as the EWT concentration increased 10-fold from 1 to 10 mg. In contrast to EWT, the standard quercetin exhibited only a 4.6-fold increase over the same concentration increment. We suggest that while the EWT extract possesses antioxidant capabilities, its activity could possibly be attributed to the concentration of active compounds within the mixture. In addition, while antioxidant activities exhibited by EWT were not as potent as the positive control, the presence of these active compounds could still contribute to the overall health benefits associated with the tonic.

While the EWT extract exhibited lower antioxidant activity compared to quercetin, its potential as an antioxidant source remains relevant and possesses antioxidant capability. The observed activity is indicative of the presence of active antioxidant compounds, even if at lower concentrations, due to the presence of numerous other constituents derived from the plant extracts in the formulation (Irawan et al., 2022).

Antimicrobial Activity

While the antimicrobial activity of EWT extract was evaluated with a disk diffusion method, susceptibility of the pathogens towards the EWT extract was assessed by measuring the clear zone (inhibition zone) surrounding the extract. Streptomycin sulfate was used as a

positive control for bacterial assay, whereas nystatin was used as a positive control for fungal assays. Table 3 shows the diameter of the inhibition zone of the EWT extract against the tested microorganisms. At the high concentration of 400 mg/mL of EWT extract, the antibacterial as well as antifungal capabilities are weaker in comparison with their respective standards, indicating the limited potential of EWT to serve as an antimicrobial agent (Table 3).

Table 2
Comparison among FRAP, DPPH and ABTS assays on EWT extract

No	Samples	FRAP (mg of $FeSO_4$ equivalent/mg of extract)		DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)
		1 mg	10 mg		
1	EWT	0.14 ± 0.03	1.57 ± 0.32	2.0691 ± 0.21	10.0069 ± 0.51
2	Quercetin	5.34 ± 1.06	24.32 ± 5.76	0.0149 ± 0.00	0.1849 ± 0.03
3	Methanol	-0.01± 0.02	-0.01 ± 0.02	Not Applicable	Not Applicable

Note. All values represent means ± SD of three replicates; IC_{50}: Half maximal inhibitory concentration

Table 3
The inhibition diameter of EWT extract against pathogenic microbes

Microorganism	Inhibition Diameter (mm)		
	400 mg/mL of EWT Extract	Streptomycin Sulfate	Nystatin
Bacteria			
Bacillus subtilis	7.3 ± 0	35.0 ± 0	nt
Streptococcus pyogenes	12.0 ± 0	35.0 ± 0	nt
Fungi			
Candida albicans	6.0 ± 0	nt	25.0 ± 0
Aspergillus niger	8.0 ± 0	nt	20.0 ± 0

Note. * nt-not tested

While the EWT extract exhibited lower antioxidant activity compared to quercetin, its potential as an antioxidant source remains relevant and possesses antioxidant capability. The observed activity is indicative of the presence of active antioxidant compounds, even if at lower concentrations, due to the presence of numerous other constituents derived from the plant extracts in the formulation (Irawan et al., 2022).

CONCLUSION

Extracts from the EWT exhibit strong COX-2 inhibition, highlighting the tonic's potential for managing inflammatory conditions with notable antioxidants, and weak antimicrobial activities, supporting its traditional use for pain relief, reduction and management of inflammation. These results have demonstrated the potential of the waist tonic to serve as a natural remedy for conditions associated with oxidative stress and inflammation, although it was found to be less effective in microbial infections. In-depth investigation of the specific bioactive compounds isolated from EWT that are responsible for these effects, and moreover, the *in vivo* work, may enhance their application in modern healthcare.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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