



# Anticancer and DNA-Protecting Potentials of *Spilanthes acmella* (Toothache Plant) Grown in Mizoram, India

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## Abstract

The toothache plant, *Spilanthes acmella* (L.) Murray, is an important culinary and medicinal plant. Here, we study its probable properties as a protective agent for DNA damage and cell proliferation, the two molecular events in oncogenesis. Plant extract was prepared by hot extraction using methanol as solvent. Various chemical tests were performed to collectively understand its free radical-scavenging activity, including 2,2-diphenyl-1-picrylhydrazyl, nitric oxide, potassium ferricyanide, hydroxyl radical, and hydrogen peroxide assays. Free radicals used or produced in these assays represent those that cause DNA damages in cells. The plant extract showed free radical-scavenging activity in all the tests and was as effective as standard drugs. Cancer cell lines such as Dalton's lymphoma ascites (DLA) and Chinese hamster lung carcinoma (V79) cells were used for anticancer assays. The plant extract showed anticancer activity only on lymphoma cells; but there was no discernible inhibition on carcinoma. The study shows that *S. acmella* is a promising source of therapeutic agent in the prevention of cancer development and DNA damage.

**Keywords:** Chinese Hamster Lung Carcinoma, Dalton's Lymphoma Ascites, Free Radical Scavenging, Medicinal Plant, MTT Assay

## 1. Introduction

*Spilanthes acmella* (L.) Murray (Asteraceae) is used in various traditional practices covering a wide range of medicinal uses. It is a small pungent plant, used for aesthetic values such as planting as ornamental herb, to cuisine. In various Asian dishes, its aerial parts are consumed either cooked or as a spice for seasoning foodstuffs. The fiery taste in the mouth and numbness it produces are the basis for its use as anticariogenic and local unaesthetic, particularly in dental care. It is for this usage that a common name "toothache plant" was given. It is also used as an anticonvulsant, antibiotic, antifungal, antiprotozoal, analgesic, antiulcer, antipyretic, antidiuretic, antiinflammatory, anthelmintic, antiviral and as an insecticide<sup>1</sup>. In Indian traditional system, it is used for the treatment of impotency and as a general aphrodisiac. It is also used in complicated clinical

conditions such as in snakebite, articular rheumatism, and tuberculosis<sup>2</sup>. Its vasorelaxant and Botox-like effects are exploited in cosmetics for the development of antiaging cream<sup>3</sup>.

A number of biologically important compounds have been reported from this plant. It is known to contain alkylamides<sup>4</sup>,  $\alpha$ - and  $\beta$ -amyrinester, isobutylamides, stigmasterol, miricilic alcohol glycosides, sitosterol, saponins and triterpenes. In addition, essential oils such as limonene,  $\beta$ -caryophyllene, (Z)- $\beta$ -ocimene, germacrene D, and myrcene have been identified<sup>5</sup>. These compounds are largely attributed to the antioxidant, antimicrobial, cytotoxic activities and other therapeutic uses of the plant<sup>1</sup>. But little is known about their specific biochemical interactions and cellular effects.

Free radicals are known for their "two-faced" nature. Being inherently produced in cellular metabolism, they

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can exert unprecedented and undesirable effects. Within the cells they act as secondary messengers and trigger an array of intracellular signalling cascades, including those which activate oncogenes and apoptotic pathways<sup>6</sup>. Hydroxyl radical particularly is deleterious and is the most powerful free radical known to science; its ability to attack and destroy virtually all kinds of cellular molecules is incredible. It is the product of molecular oxygen from successive monovalent reduction during normal cell metabolism. It can insinuate a chain of chemical reactions that intervene different signalling pathways. It can also attack fatty acids, the structural backbone of cell membranes, bringing about lipid peroxidation and eventual cell death<sup>7</sup>. It can directly impair DNA bases to initiate mutation, even leading to DNA strand breaks. It is by this molecular mechanism that cancer cells developed, and different cancers share the basic etiological characteristics<sup>8</sup>. Hence, this study was carried out to investigate the DNA-protecting and anticancer potentials of *Spilanthes acmella*.

## 2. Materials and Methods

### 2.1 Plant Extract

Flowering *S. acmella* with its aerial parts were collected from agricultural fields at Ngopa village, northeast of Mizoram, India. The specimens (accession no. PUC-A-17-1) were identified at the Botanical Survey of India (BSI), Shillong, Meghalaya. It was remarked that the specimens have notable variations from the type specimens in having curly-edged leaves, and unicolour, dome-shaped flowers, in comparison to the type specimens. The aerial parts were dried under shade for a month, and pulverised using electric grinder. The plant extract was prepared in a Soxhlet apparatus using methanol. The solvent was completely removed and recovered from the extract using a rotary vacuum evaporator (Buchi Rotavapor® R-215). The final semi-solid extract was refrigerated at 4°C for further use.

### 2.2 Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, sodium dodecyl sulphate, RPMI-1640 media and trypan blue were procured from Sigma Aldrich Chemical Co., Kolkata, India. Other chemicals were

procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

### 2.3 Cancer Cell Lines

Chinese hamster carcinoma cell, V79 (ATCC® CCL-93™), was procured from National Centre for Cell Science, Pune, India. Dalton's lymphoma ascites (DLA) was obtained from serial transplantation in Swiss albino mice.

### 2.4 DPPH Scavenging Assay

1 ml of 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was mixed with 3 ml of the plant extract (prepared in 10, 20, 40, 60, 80, and 100 µg/ml). After incubating at 37±1°C for 30 minutes, absorbance was taken at 517 nm in a UV-visible spectrophotometer (Evolution™, Thermo Scientific). A control consisted of DPPH solution, and standard reference was butylated hydroxytoluene (BHT). The percentage of inhibition was calculated using the formula

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 2.5 NO Scavenging Assay

1 ml each of 10, 20, 40, 60, 80, and 100 µg/ml of the plant extract was mixed with 2 ml of 10 mM sodium nitroprusside. Equal amount and concentrations of Griess reagent (a mixture of sulfanilamide, N-1-naphthylethylenediamine dihydrochloride and phosphoric acid) were used as standard. After incubating at 30°C for 2 hours, the mixtures were treated with 0.5 ml of Griess reagent. Absorbance was taken at 550 nm. The percentage of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 2.6 Potassium Ferricyanide Scavenging Assay

1 ml of different concentrations (viz. 10, 20, 40, 60, 80, and 100 µg/ml) of the plant extract were mixed with 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide (PFC). Equal concentrations of ascorbic acid were used

as standard. The mixture was incubated at 50°C for 30 minutes and the reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid. After centrifugation at 3000 rpm for 10 minutes, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water, and 0.5 ml of 0.1% ferric chloride solution. Absorbance was taken at 700 nm. The percentage of inhibition was deduced as follows:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

## 2.7 Hydroxyl Radical Scavenging Assay

1 ml of different concentrations (viz. 10, 20, 40, 60, 80, and 100 µg/ml) of the plant extract were mixed with 0.1 ml of 1 mM EDTA, 0.01 ml of 10 mM FeCl<sub>3</sub>, 0.1 ml of 10 mM H<sub>2</sub>O<sub>2</sub> and 0.36 ml of 10 mM deoxyribose. Similar mixture was prepared for ascorbic acid as standard. About 0.33 ml of phosphate buffer and 0.1 ml of 0.1 mM ascorbic acid were added and then incubated at 37°C for 1 hour. 1 ml of the mixture was treated with 1 ml of trichloroacetic acid (TCA) and 1 ml of 0.5% thiobarbituric acid (TBA). They were heated at 80°C for 10-20 minutes until pink chromagen developed. After letting them cool down, absorbance was taken at 532 nm. The results were calculated as percent inhibition of the deoxyribose attack using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

## 2.8 Hydrogen Peroxide Radical Scavenging Assay

40 mM of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was prepared in phosphate buffer and different concentrations of extract (viz. 10, 20, 40, 60, 80, and 100 µg/ml) and standard (ascorbic acid) were added. After 10 minutes, the absorbance was taken at 230 nm. The percentage of hydrogen peroxide was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

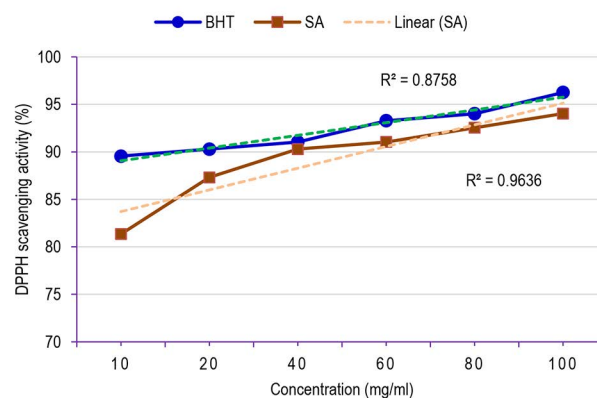
## 2.9 MTT Assay

Approximately 5000 cells from each of DLA (Dalton's lymphoma ascites) and V79 (Chinese hamster lung fibroblast) cell lines were seeded into 96-well microplates. They were mixed with 10, 20, 40, 60, 80, and 100 µg/ml of the plant extract. A control without the extract was maintained. They were incubated at 37°C for 48 hours under 5% CO<sub>2</sub> and 95% humidity. 20 µl/ml of methylthiazolyldiphenyl-tetrazolium (MTT) bromide in phosphate-buffered saline was added to each well and were incubated for another 2 hours. Formazan crystals were formed, to which 100 µl of MTT lysis buffer was added to dissolve them. The cultures were further incubated overnight. Absorbance was taken at 570 nm in a microplate reader (SpectraMax® M2). The inhibition percentage of the cells was calculated by the following equation:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

## 2.10 Statistical Analyses

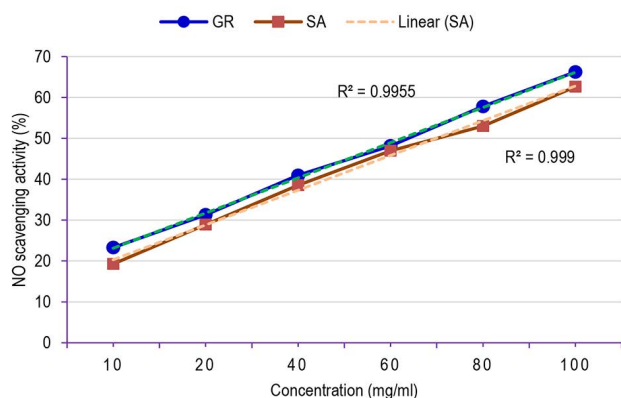
Data were analysed with OriginPro-8 (OriginLab Corporation, Northampton, USA) and Microsoft Excel 2016. Linear regression analysis was used to extrapolate IC<sub>50</sub> values from concentration-effect curves. For cell inhibition assay, Student's *t*-test was employed, and significance was taken at *p* ≤ 0.05.



**Figure 1.** Free radical-scavenging activity of *S. acmella* on 2,2-diphenyl-1-picrylhydrazyl (DPPH). BHT = butylated hydroxytoluene; SA = *S. acmella* extract.

### 3. Results

The free radical scavenging activity of *S. acmella* extract using DPPH as a substrate is shown in Figure 1. At varying concentrations, such as 10, 20, 40, 60, 80, and 100 µg/ml, both the standard butylated hydroxytoluene (BHT) and the plant extract exhibited concentration-dependent scavenging activity of DPPH. BHT indicated higher activity than that of the plant extract at all concentrations tested. For BHT, the inhibition ranges from 89.55 to 96.26 % for the lowest and highest concentrations respectively. While it ranged from 81.34 to 94.03 % for that of the plant extract. The overall activity is thus quite comparable.

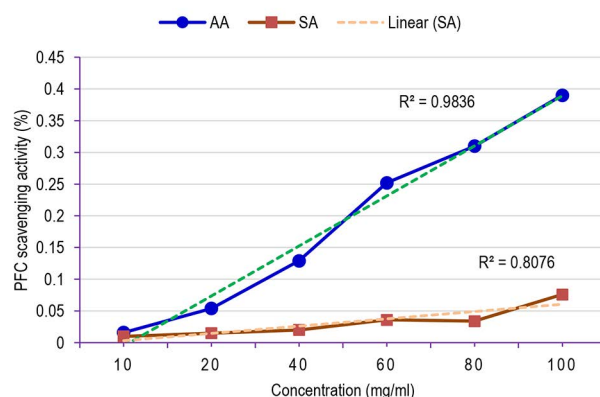


**Figure 2.** Nitric oxide (NO) scavenging activity of *S. acmella*. GR = Griess reagent; SA = *S. acmella* extract.

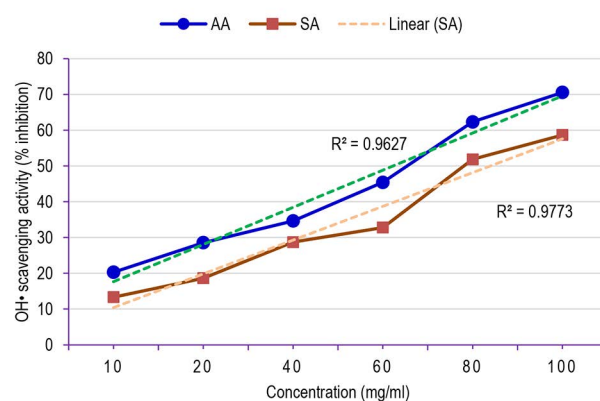
*S. acmella* extract showed concentration-dependent activity against nitric oxide radicals using sodium nitroprusside reaction (Figure 2). Griess reagent was used as the standard reference, which indicated higher activity at corresponding concentrations, but the trend of activity was similar. Highest activity at 100 µg/ml was 66.26% for the standard compound, and 62.65% for the plant extract. The overall inhibitory concentration ( $IC_{50}$ ) determined from the standard graph was 4.121 µg/ml for the standard, and 4.492 µg/ml for the plant extract. The plant extract showed almost an equal potency as the standard antioxidant in scavenging NO.

*S. acmella* extract showed concentration-dependent scavenging activity of potassium ferricyanide (Figure 3). However, the effect was relatively low compared to that of the standard drug ascorbic acid.

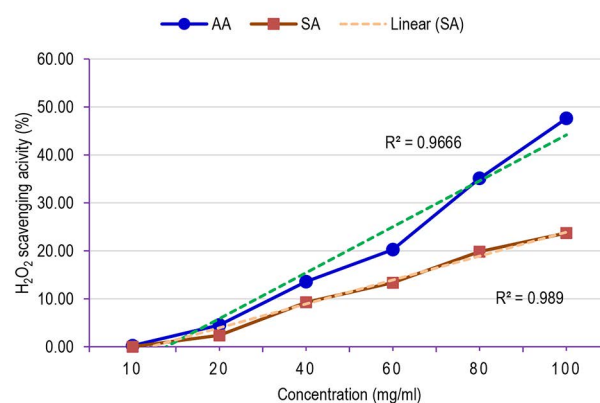
Inhibition at the highest concentration (100 µg/ml)



**Figure 3.** Potassium ferricyanide (PFA) scavenging activity of *S. acmella*. AA = ascorbic acid; SA = *S. acmella* extract.



**Figure 4.** Hydroxyl radical (OH•) scavenging activity of *S. acmella*. AA = ascorbic acid; SA = *S. acmella* extract.



**Figure 5.** Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging activity of *S. acmella*. AA = ascorbic acid; SA = *S. acmella* extract.

was 0.39% for ascorbic acid, but only 0.076% for the plant extract.

The scavenging activity of *S. acmella* extract on hydroxyl radicals in comparison with ascorbic acid is presented in Figure 4. Their activities were quite comparable. The plant extract showed lowest activity of 13.34% and higher cavity of 58.70%; while ascorbic

Significant inhibition ( $p < 0.05$ ) was found at the concentration 40  $\mu\text{g/ml}$  and above. However, the  $\text{IC}_{50}$  was 147.547  $\mu\text{g/ml}$ . In contrast, the plant extract did not have significant cytotoxicity ( $p > 0.05$ ) on V79 cells, and instead seems to promote their proliferation at higher concentration. This may suggest that the plant extract is preferentially active on specific cancer cells.

**Table 1. MTT assay of *S. acmella* extract on two cancer cell lines.**

Cell line	Concentration ( $\mu\text{g/ml}$ )	Absorbance at 570 nm (mean $\pm$ SEM)	% inhibition	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )
Control	0	0.682 $\pm$ 0.022	0.000	
	10	0.700 $\pm$ 0.014	28.574*	
	20	0.749 $\pm$ 0.009	23.610*	
Murine carcinoma (V79)	40	0.847 $\pm$ 0.013	13.581*	54.341
	60	0.930 $\pm$ 0.018	5.150*	
	80	0.952 $\pm$ 0.035	2.941	
	100	1.040 $\pm$ 0.025	-6.068	
	10	3.397 $\pm$ 0.041	-7.528	
	20	3.256 $\pm$ 0.044	-3.054	
	40	3.051 $\pm$ 0.233	3.424*	
Dalton's lymphoma ascites	60	2.907 $\pm$ 0.075	7.971*	147.547
	80	2.489 $\pm$ 0.030	21.232*	
	100	2.120 $\pm$ 0.297	32.911*	

\*Significantly different at  $p \leq 0.05$  in comparison to control.

acid showed 20.32% and 70.58% respectively. The  $\text{IC}_{50}$  of the extract on hydroxyl radicals was calculated as 5.193  $\mu\text{g/ml}$ .

*S. acmella* extract showed  $\text{H}_2\text{O}_2$  scavenging activity, but its potency is much lower than that of the standard ascorbic acid (Figure 5). At the highest concentration of 100  $\mu\text{g/ml}$ , the plant extract showed 23.71% of scavenging activity; while that for ascorbic acid was double of that, i.e. 47.63%. The  $\text{IC}_{50}$  of the plant extract was 5.278  $\mu\text{g/ml}$ .

MTT assay using murine carcinoma (V79) and Dalton's lymphoma ascites (DLA) cells showed that the plant extract had cell-selective activity as presented in Table 1. It was mostly effective in inhibiting the proliferation of DLA cells, with a concentration-dependent activity.

## 4. Discussion

During aerobic metabolism in eukaryotic cells, especially in mitochondrial respiratory chain reaction by which the energy-source molecule ATP is synthesised, the so-called oxidants or reactive oxygen species (ROS) are synthesised as normal by-products. Such ROS include free radicals such as oxygen radical ( $\text{O}_2\bullet\bullet$ ), superoxide ( $\text{O}_2\bullet^-$ ), hydroxyl radical ( $\text{OH}\bullet$ ), peroxy ( $\text{ROO}\bullet$ ) and lipid peroxy ( $\text{LOO}\bullet$ ). In low oxygen condition, the mitochondrial electron transfer system also produce nitric oxide (NO), which can generate nitrogen-based free radicals called reactive nitrogen species (RNS) such as  $\text{NO}\bullet$  and nitrogen dioxide ( $\text{NO}_2\bullet$ ). Although other metabolic products such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ozone ( $\text{O}_3$ ), singlet oxygen ( $^1\text{O}_2$ ), hypochlorous



acid (HOCl), hypobromous acid (HOBr), nitrous acid (HNO<sub>2</sub>), peroxyxynitrite (ONOO<sup>-</sup>), nitrosyl cation (NO<sup>+</sup>), nitroxyl anion (NO<sup>-</sup>), dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), dinitrogen tetraoxide (N<sub>2</sub>O<sub>4</sub>), nitronium (nitryl) cation (NO<sub>2</sub><sup>+</sup>), organic peroxides (ROOH), aldehydes (HCOR), peroxyxynitrite (ONOOH), and lipid peroxide (LOOH) are not free radicals, but they are still oxidants due to their ability to induce free radical reactions<sup>9</sup>.

The unusual behaviour of these chemicals is that their free electrons have a tendency to react (by donating or accepting other's electrons) with fundamental biomolecules such as lipids, proteins, and nucleic acids<sup>10</sup>. They therefore play a major role in the pathogenesis of a variety of chronic diseases including as arthritis, autoimmune disorders (multiple sclerosis), cancer, cardiovascular diseases (atherosclerosis and hypertension), diabetes mellitus, neurological diseases (Parkinson's disease, Alzheimer's disease), respiratory diseases (asthma), cataract development, rheumatoid arthritis and in various cancers (colorectal, prostate, breast, lung, bladder cancers)<sup>8</sup>.

When the free radical-induced signalling pathways had gone awry, the endogenous antioxidants present in the cells are insufficient to prevent the detrimental effects. As such, the body has to be supplemented with exogenous antioxidants<sup>11</sup>. Natural antioxidants particularly from fruits and vegetables are the main and best sources. Plant-derived compounds such as polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C)<sup>12</sup>. The potentiality of these plants in the prevention and prophylaxis of debilitating diseases due to oxidative has been experimentally warranted<sup>13</sup>.

Various investigations on the potentiality of *S. acmella* in free radical scavenging and anticancer activities in this study indicate that the plant is of significant pharmacological value. Its ability to prevent and intercept DNA damage is evidenced by its activity against important free radicals. This is notable because natural products are the primary and principle sources of exogenous antioxidants. For instance, quercetin (3,3',4',5,7-pentahydroxyflavone), a flavonoid glycoside belonging to phenolic compounds, present in fruits, vegetables, and wine is one of the most widely used natural antioxidants for the treatment of a range of infection, metabolic and inflammatory disorders. In

addition, it has been a tradition to use quercetin as a standard compound in the estimation of antioxidant activity itself<sup>14</sup>.

MTT assay reflects the enzymatic activity of mitochondrial dehydrogenases in intact cells. These oxidative enzymes breakdown MTT to purple-coloured formazan crystals. The resultant colour intensity reflects the degree of enzyme activity, and thus, cell viability<sup>15</sup>. Mitochondria are the active sites of cellular redox reactions where ROS are produced; hence, they are referred to as a "necessary evil" in the life of eukaryotic cells<sup>16</sup>. The ability of *S. acmella* to induce cytotoxicity in a cell-specific manner is an important information. The plant extract is most potent on the lymphoma (Dalston's lymphoma ascites) cells with an IC<sub>50</sub> of 147.547 µg/ml, while it has no effect on lung carcinoma (V79). This implies that the bioactive compound(s) of the plant act on the cell signalling molecules that are involved in regulating the interphase stage of the cell cycle.

## 5. Conclusion

*S. acmella* extract was tested for its efficacy on oxidants using various chemical assays. Inherent DNA damages are due to oxidative stress that is induced by different free radicals. This study shows that *S. acmella* is able to protect DNA damages by scavenging the important oxidants. The plant extract also showed anticancer activity on Dalton's lymphoma ascites. These findings posit a possibility that the plant contains unique bioactive molecule(s) that can prevent DNA damage and cell cycle disruption, and provide more challenges for further investigations.

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