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# Analysis of the mechanisms underlying the analgesic effects of the extracts of *Phyllanthus amarus & Phyllanthus fraternus*

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## Abstract

A great number of preclinical and clinical studies have not only confirmed but have also extended the medicinal uses of species of the genus *Phyllanthus* mentioned in traditional medicine. We have examined some of the mechanisms underlying the analgesic effects of the extracts of *Phyllanthus amarus* and *Phyllanthus fraternus* against formalin-induced nociception in mice. In addition, we also investigated the action of both the species against capsaicin-mediated pain. 20 µl of 2.5% formalin (0.92% formaldehyde), made up in phosphate-buffer solution, was injected intraplantarly in the right hindpaw. Animals were pre-treated with the extracts of *Phyllanthus amarus* and *Phyllanthus fraternus* and with specific receptor antagonists for evaluating the mechanisms of analgesic activity. 20 µl of capsaicin (1.6 µg/paw) was injected intraplantarly in the right hind paw. Animals were treated with the extracts of *P. amarus* and *P. fraternus* intraperitoneally (1-30 mg/kg) 30 min before, or orally (25-200 mg/kg) 60 min before capsaicin injection. Both the adrenergic receptor antagonists prazosin and yohimbine (0.15 mg/kg, i.p.) had no effect on the antinociceptive action caused by extracts of *P. amarus* (30 mg/kg, i.p.) and *P. fraternus* (30 mg/kg, i.p.). Treatment of animals with L-arginine (600 mg/kg) had no significant effect against the analgesic properties of *P. amarus* and *P. fraternus*. The extracts of *Phyllanthus amarus and Phyllanthus fraternus* given either intraperitoneally or orally caused marked and dose-related inhibition of capsaicin-induced pain. The current study suggest that their antinociceptive action is unrelated to central depressor action, interaction with α-adrenergic receptor or interaction with L-arginine nitric oxide pathway.

**Key words:** Adrenergic, L-arginine, formalin, capsaicin, *Phyllanthus amarus, Phyllanthus fraternus.*

## Introduction

The plants belonging to the genus Phyllanthus (Euphorbiaceae) are widely distributed throughout tropical and subtropical countries (Calixto *et al.,*1998; Bagalkotkar *et al.,*2006 and Patel *et al.,*2011). These plants are used in folk medicine for treatment of several diseases, such as disturbances of kidney and bladder calculi, intestinal infections, diabetes and hepatitis B virus. A great number of preclinical and clinical studies have not only confirmed but have also extended the medicinal uses of species of the genus Phyllantus mentioned in traditional medicine (Calixto *et al.,*1998; Bagalkotkar *et al.,*2006 and Patel *et al.,*2011).

Previous studies showed that the extracts of genus Phyllanthus revealed potent, dose related and long-lasting antinociceptive properties when tested in several models of pain and nociception in experimental animals (Santos *et al.,*1995a; Santos *et al.,*1995b; Santos *et al* 2000; Kassuya *et al.,*2003 and Kiemer *et al.,*2003). However, the mechanisms that underlying their analgesic effect still remains unclear. In the present study, we therefore attempted to examine some of the mechanisms involved in the antinociceptive profile of the extracts of *Phyllanthus amarus* Schum and Thonn and *Phyllanthus fraternus* Webster against formalin-induced nociception in mice. Additionally, we also investigated the effect of extracts of both *P. amarus* & *P. fraternus* against capsaicin-induced pain in mice.

## Materials and methods

### *Plant material*

*Phyllanthus amarus* Schum and Thonn and *Phyllanthus fraternus* Webster family- Euphorbiaceae were obtained from different places in Karad Western Maharashtra. The plant species were identified and authenticated by Botanical survey of India, Pune [Reference No:BSI/WC/Tech./2012/644].

### *Preparation of the P. fraternus extract*

The dried leaves, stems and roots of *P. fraternus* was minced and extracted with 70% ethanol-water in the proportion of 70:30, being stirred and macerated at room temperature (22-28°C) for 15 days. The ethanol was evaporated and the extract (yield 5-7%) was concentrated to the desired level and stored in a refrigerator. The extract was dissolved in DMSO [dimethyl sulphoxide] to the desired concentration just before use.

### *Extracts of Phyllanthus amarus*

The standardized extract of *Phyllanthus amarus* whole plant (water extract) Reference No: SR/KN/CL/1/2012-L12030241, was procured as a gift sample from Chemiloids Ltd., Vijayawada. While the standardized methanolic extract of *Phyllanthus amarus* leaf (Methanol extract contains >2.5% of Phyllanthin and Hypophyllanthin) Report No: FP1112042- PA/11LOT05 and the standardized hydro methanolic extract of *Phyllanthus amarus* leaf (60% Methanol Hydroalcoholic extract contains >5% of Corilagen) - Report No: FP1102034 -PA/11LOT/02 were procured as a gift sample from Natural Remedies Pvt. Ltd., Bangalore.

### *Experimental Animals*

Swiss albino mice (25-30 g) were used for the study. The animals were maintained under standard environmental conditions and were fed with standard diet and water *ad libitum*. Food and water were freely available throughout the experiments.

A prior approval [Approval number- GCOPK/2011-12/CPCSEA/616] was obtained from the Animal Ethics Committee of Govt. College of Pharmacy, Karad (GCOPK) for the study [Protocol Reference No: CPCSEA-IAEC/2011-NOV/01 & CPCSEA-IAEC/2012-MAR/01]. GCOPK is registered under Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Govt. of India, CPCSEA Registration no- 209/GO/a/2000/ CPCSEA.

### *Drugs*

The drugs used were Formalin, Capsaicin and L-arginine (Loba chemicals Mumbai), Prazosin and Yohimbine (Sun Pharma). All other reagents used were of the highest grade of purity. All drugs and extracts were dissolved in DMSO just before use.

Animals from the same strain were slightly anaesthetized with ether and 20 microlitre of 2.5% formalin (0.92% formaldehyde) made up in phosphate buffer solution was injected under the paw surface of the right hind paw. The control and treated animals were observed for 0-30 min after formalin injection. The amount of time spent licking the injected paw was timed and was considered as indicative of pain (Santos *et al.,*1995b & Santos *et al.,* 2000). The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15-30 min after formalin injection (second phase), representing the neurogenic and inflammatory pain responses, respectively. Animals were treated with DMSO (10 ml/kg, i.p.) or with standardized aqueous extract of *P. amarus* whole plant (PAAE), standardized methanolic extract of *P. amarus* leaf (PAME) and the standardized hydro methanolic extract of *P. amarus* leaf (PAHME) at dose of 30 mg/kg, i.p. and the standardized hydro ethanolic extract *P. fraternus* (PFHEE) (30 mg/kg, i.p.) 30 min before formalin injection.

After intraplantar injection of formalin, the animals were immediately placed into a glass cylinder 20 cm in diameter, and the time spent licking the injected paw (first and second phase of formalin test) was determined.

### *Effect on Adrenergic system*

To assess the possible participation of α-adrenergic receptors, animals were pretreated with yohimbine (α2 antagonist, 0.15 mg/kg, i.p.) or with prasozin (α1 antagonist, 0.15 mg/kg, i.p.) 15 min before administration of phyllanthus extracts. The pain response caused by formalin injection was analysed 30 min after the administration of the phyllanthus extracts (Santos *et al.,*1995b & Santos *et al.,* 2000).

### *Effect on nitric oxide L-arginine pathway*

In separate experiments, we investigated the possible participation of nitric oxide L-arginine pathway on the analgesic effect caused by extracts of the studied species of Phyllanthus. The animals were pretreated with L-arginine (600 mg/kg, i.p.), and after 15 min they were treated various phyllanthus extracts. The pain caused by the first and second phase of formalin injection was analysed 30 min after treatment of animals with the phyllanthus extracts.

In the capsaicin test, 20 min before testing, the animals were placed individually in transparent glass cylinders 20 cm in diameter, which also served as the observation chambers. After the adaptation period, 20 µl of capsaicin (1.6 µg per paw) was injected under the skin of the dorsal surface of the right hind paw using a microsyringe with a 26-gauge needle. The procedure used was similar to that described previously with

minor modifications. The animals were observed individually for 5 min after capsaicin injection. The amount of time spent licking the injected paw was timed and was considered as indicative of pain (Santos *et al.,*1995b & Santos *et al.,* 2000).

Animals were treated with standardized aqueous extract of *P. amarus* whole plant (PAAE), standardized methanolic extract of *P. amarus* leaf (PAME), standardized hydro methanolic extract of *P. amarus* leaf (PAHME) and the standardized hydro ethanolic extract *P. fraternus* (PFHEE) at a dose of (1-30 mg/kg) intraperitoneally 30 min before or (25-200 mg/kg) orally 60 min before capsaicin injection. Control animals received a similar volume of DMSO (10 ml/kg) either intraperitoneally or orally.

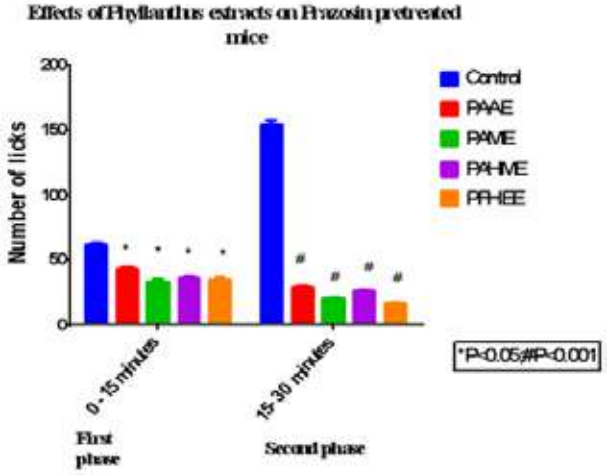
## Statistical analysis

All the statistical calculation were performed using Graphpad Prism software version 6.01, © 1992-2012. Results were presented as means ± SEM (standard error of mean), except the $ID_{50}$ that are presented as geometric means accompanied by their respective 95% confidence limits. Statistical significance between groups was calculated by means of analysis of variance followed by Newman-Kuels' multiple comparison test. $P < 0.05$ was considered significant. When appropriate, the ID50 (i.e., the dose of extracts that reduced responses by 50% relative to control values) were estimated for individual experiments using the least-squares method.

## Results

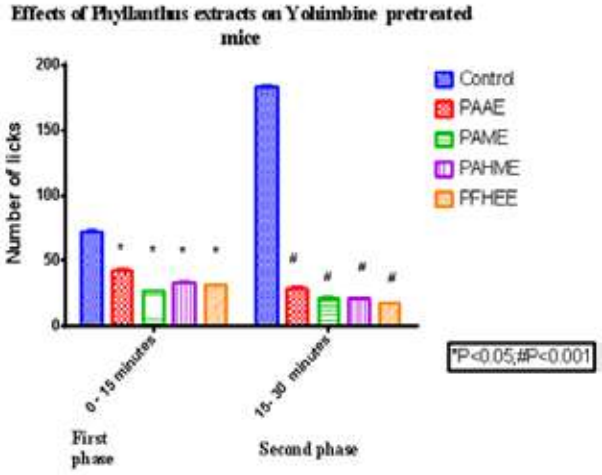
The antinociceptive action of *P. amarus* and *P. fraternus* extracts was unaffected by the pretreatment of animals with α-adrenergic antagonists, prazosin (0.15 mg/kg, i.p.) or yohimbine (0.15 mg/kg, i.p.) 30 min before formalin test (Figures 1& 2).

**Fig. 1** Effects of Phyllanthus extracts on Prazosin pretreated mice




Each group represents the mean of four experiments and vertical bars indicate SEM. *P< 0.05; #P< 0.001 compared with corresponding control value PAAE: *Phyllanthus amarus* aqueous extract of whole plant, PAME: *Phyllanthus amarus* methanolic extract leaf, PAHME: *Phyllanthus amarus* hydro methanolic extract of leaf and the PFHEE: *Phyllanthus fraternus* hydro ethanolic extract of whole plant.

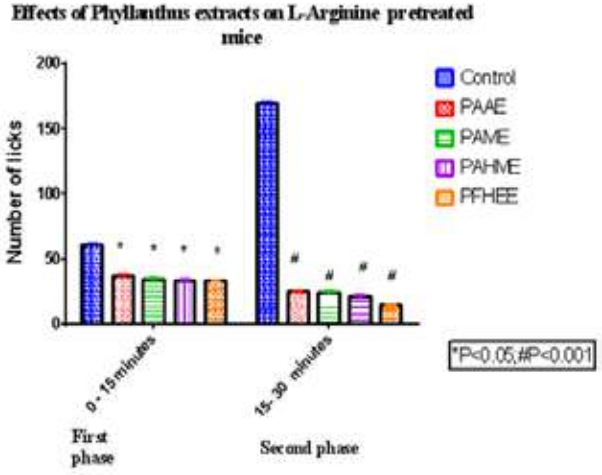
**Fig. 2** Effects of Phyllanthus extracts on Yohimbine pretreated mice




Each group represents the mean of four experiments and vertical bars indicate SEM. *P< 0.05; #P< 0.001 compared with corresponding control value PAAE: *Phyllanthus amarus* aqueous extract of whole plant, PAME: *Phyllanthus amarus* methanolic extract leaf, PAHME: *Phyllanthus amarus* hydro methanolic extract of leaf and the PFHEE: *Phyllanthus fraternus* hydro ethanolic extract of whole plant.

The treatment of animals with L-arginine (600 mg/kg, i.p.) a precursor of nitric oxide synthase, did not affect the antinociceptive action of *P. amarus* and *P. fraternus* extracts (Figure 3).

**Fig. 3** Effects of Phyllanthus extracts on L-Arginine pretreated mice.

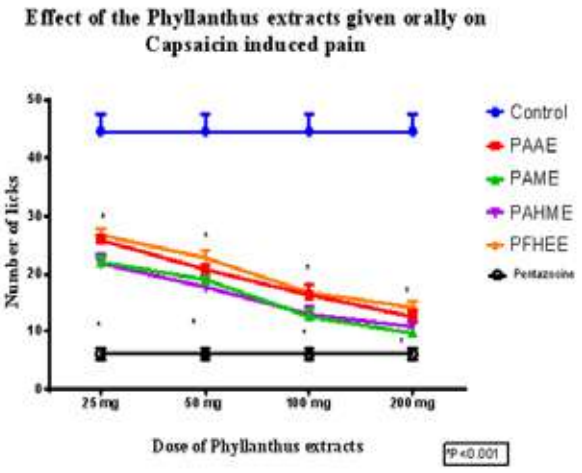



Each group represents the mean of four experiments and vertical bars indicate SEM. *P< 0.05; #P< 0.001 compared with corresponding control value PAAE: *Phyllanthus amarus* aqueous extract of whole plant, PAME: *Phyllanthus amarus* methanolic extract leaf, PAHME: *Phyllanthus amarus* hydro methanolic extract of leaf and the PFHEE: *Phyllanthus fraternus* hydro ethanolic extract of whole plant.

Given orally (25-200 mg/kg), the *P. amarus* and *P. fraternus* extracts also caused dose-related inhibition of capsaicin induced nociception (Figure 4). The maximal inhibitions for PAME, PAHME, PAAE & PFHEE were 78.09, 75.84, 69.78 and 67.97 % respectively (Figure 6).

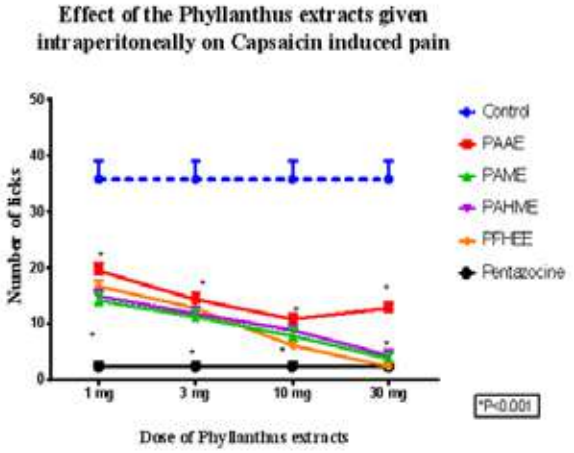
When tested in the capsaicin algesic model, the extracts of Phyllanthus species (1-30 mg/kg, i.p.) 30 min before caused marked and dose-related inhibition of capsaicin-induced neurogenic pain (Figure 5). The maximal inhibitions for PAME, PAHME, PAAE & PFHEE were 89.51, 88.18, 72.72 and 93.70 % respectively (Figure 7).

**Fig.** 4 Effect of the Phyllanthus extracts given orally on Capsaicin induced pain




Each group represents the mean of four experiments and vertical bars indicate SEM. *P < 0.001 compared with corresponding control value PAAE: *Phyllanthus amarus* aqueous extract of whole plant, PAME: *Phyllanthus amarus* methanolic extract leaf, PAHME: *Phyllanthus amarus* hydro methanolic extract of leaf and the PFHEE: *Phyllanthus fraternus* hydro ethanolic extract of whole plant.

**Fig. 5** Effect of the Phyllanthus extracts given intraperitoneally on Capsaicin induced pain




Each group represents the mean of four experiments and vertical bars indicate SEM. *P < 0.001 compared with corresponding control value PAAE: *Phyllanthus amarus* aqueous extract of whole plant, PAME: *Phyllanthus amarus* methanolic extract leaf, PAHME: *Phyllanthus amarus* hydro methanolic extract of leaf and the PFHEE: *Phyllanthus fraternus* hydro ethanolic extract of whole plant.

**Fig. 6** % Inhibitory effect of the Phyllanthus extracts given orally against the capsaicin induced pain in mice.

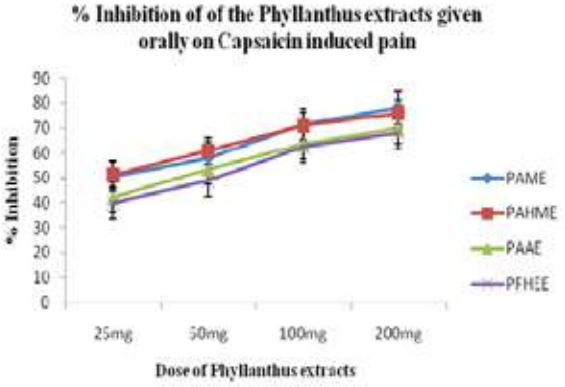
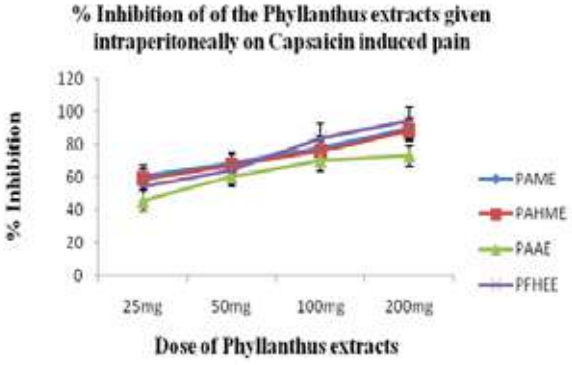



**Fig. 7** % Inhibitory effect of the Phyllanthus extracts given intraperitoneally against the capsaicin induced pain in mice.



## Discussion

### *Assessment of Adrenergic system*

It has been reported that both $\alpha_1$ and $\alpha_2$ selective agonists, phenylephrine and clonidine, when injected systemically, caused a pronounced analgesic effect against several models of pain. These effects being selectively antagonized by systemic administration of $\alpha_1$ and $\alpha_2$ selective receptor antagonists, respectively. Our results showed that the systemic treatment of animals with $\alpha_1$ and $\alpha_2$ selective receptor antagonists, prazosin and yohimbine, did not significantly affect the analgesic effect caused by extracts of either *P. amarus* and *P. fraternus* when analysed against the two phases of the formalin test. Therefore, these results suggest that the $\alpha_1$ and $\alpha_2$ adrenergic system is unlikely to be involved in the analgesic effect of the Phyllanthus species.

### *Assessment of nitric oxide L-arginine pathway*

Santos *et al.,*(1995B 2000) have demonstrated that nitric oxide synthase inhibitor N(G)-nitro-L-arginine (L-NOARG), given systemically, markedly antagonizes the second phase of the formalin induced nociception, these effects being selectively reversed by intraperitoneal injection of the nitric oxide precursor, L-arginine. However, the same treatment with L-arginine failed to reverse the analgesic effect caused by the extracts of *P. amarus* and *P. fraternus*. These findings support the notion that the L-arginine-derived or a nitric oxide-related pathway appears not to be involved in the antinociceptive action of the Phyllanthus plant species.

### *Capsaicin-induced pain*

We also investigated whether the extracts of two species of Phyllanthus might antagonize capsaicin induced nociception in mice, aalgesic model proposed as being useful in evaluating neurogenic pain and in investigating the possible antinociceptive effect of mediated by tachykinin receptor antagonism. As expected, the extracts of *P. amarus* and *P. fraternus*. given intraperitoneally or orally, caused dose-related and potent analgesic effect in the capsaicin model. Interestingly, the extracts of both the species of Phyllanthus caused similar analgesic effect in the capsaicin test, the extracts being about 8 to 10 fold less potent when they are administered orally. Furthermore, the potent antinociceptive action of both Phyllanthus extracts against the neurogenic pain response caused by capsaicin in mice strongly suggests that the active compound(s) present in these plants may be acting through interaction with tachykinin pathway.

### *Analysis of other mechanisms of Phyllanthus species*

The previously reported results show that the active principles present in some plants belonging to the genus Phyllanthus, such as *P. tenellus, R corcovadensis, R urinaria, P. niruri and R sellowianus*, exhibit potent and long-lasting systematic analgesic effect when analysed in two models of neurogenic and inflammatory pain. (Calixto *et al.,*1998, Bagalkotkar *et al.,*2006 and Patel *et al.,*(1995b 2011)

Santos *et al.,*have examined some of the mechanisms underlying the analgesic effects of the hydroalcoholic extracts (HE) of *Phyllanthus urinaria* and *Phyllanthus niruri* against formalin-induced nociception in mice. In addition, they also investigated the action of both HEs against capsaicin-mediated pain.

The hydroalcoholic extract (HE) of the four species of Phyllanthus, given intraperitoneally, produced significant inhibition of acetic acid-induced abdominal constrictions, with mean ID50 values of 0.3, 1.8, 7.4 and 26.5 mg/kg for *Phyllanthus amarus, Phyllanthus orbiculatus, Phyllanthus fraternus* and *Phyllanthus stipulatus*, respectively (Santos *et al.,* 2000). In the formalin test, the four species of Phyllanthus, also produced graded inhibition against both phases of formalin-induced licking, being more active in relation of the late phase. The HE of the Phyllanthus species elicited significant inhibition of the capsaicin-induced neurogenic pain, with mean ID50 values of 8.9, 6.7, >30 and ~30 mg/kg for *P. amarus, P. fraternus, P. stipulatus and P. orbiculatus*, respectively. Given orally all HE of the Phyllanthus species were less potent and efficacious than when given by intraperitoneal route (Santos *et al.,* 2000).

Candida *et al.*,(2003) have investigated the anti-allodynic and anti-oedematogenic effects of the hexanic extract, lignan-rich fraction and purified lignans from, *Phyllanthus amarus*, in the inflammatory and neuropathic models of nociception (Kassuya *et al.,*2003).

Kiemer *et al.,* (2003) have investigated potential anti-inflammatory properties of standardized P. amarus extracts concerning a potential influence of P. amarus on endotoxin induced nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and cytokine production *in vivo* and *in vitro* (Kiemer *et al.,*2003). We have postulated from our observations that the anti-inflammatory activity of Phyllanthus species could be due to its membrane stabilizing action and inhibition of protein denaturation (Chopade *et al.,*2012).

The mechanisms responsible for their analgesic effect still remain unclear, but the current study and also the previous data suggest that their antinociceptive action is unrelated to central depressor action, release of endogenous opioids or glucocorticoids, interaction with serotonin, α-adrenergic receptor or interaction with L-arginine nitric oxide pathway.

## Conclusion

In the present study we attempted to characterize further the mechanisms responsible for the analgesic action of *P. amarus* & *P. fraternus* in formalin-induced pain in mice by the use of selective adrenergic receptor antagonists and precursor of nitric oxide synthase. In addition, we also analyzed the analgesic effect of the extracts of these two species against capsaicin induced pain. The findings of the study strongly suggest that the analgesic effects of the active principle(s) present in these plants might result, at least in part, from their interactions with the tachykinin system and prostaglandins synthesis.

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