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Betulinic acid: A potent insect growth regulator from *Ziziphus jujuba* against *Tribolium confusum* [Duval]

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ABSTRACT

Betulinic acid, a terpenoid isolated from the bark of Ziziphus jujuba [Rhamnaceae] exhibited growth regulating activity against the stored grain pest Tribolium confusum [Duval] [Coleoptera: Tenebrionidae]. The fifth instar, sixth instar larvae and pupae were treated with 1µg/µl of Betulinic acid. Interference in moulting process, ecdysial failure and blockage of adult emergence were the important morphogenetic abnormalities observed which resulted in the formation of permanent larvae, larval-pupal intermediates, abnormal pupae, pupal-adult intermediates and deformed adults. These resultant forms were ruled out from further development and reproduction. Our results suggest that Betulinic acid shows an effective insect growth regulating activity and exhibits great promise in suppressing the population of stored grain pest, Tribolium confusum.

Key words: Betulinic acid, *Tribolium confusum*, Insect growth regulator, Morhogenetic abnormalities, ecdysial failure.

INTRODUCTION

The growing awareness of hazards associated with the large scale use of synthetic insecticides has evoked a worldwide interest in pest control agents of plant origin [Feeny, 1992]. These pesticides aim to exploit the insect hormonal systems, affecting metamorphosis and resulting in deformed progenies incapable of further reproduction. They are comparatively less toxic, biodegradable, have no residual effects and hold great promise as insect growth regulators [Vardhini *et al.* 2001].

Most of the insect growth regulators [IGRs] with juvenile hormone activity which have been synthesized and reported to date are derived from terpenes and sesquiterpenes [Pallos *et al*. 1976].

Terpenes are a wide spread group of natural compounds with considerable practical significance. The most important group of terpenes are triterpenes, triterpene glycosides and other triterpenoids, representing one of the numerous classes of natural compounds abundant in plant kingdom. There are atleast 4000 known triterpenes [Jiri Patocka, 2003].

There is growing interest in natural triterpenoids because of their wide spectrum of biological activities [Dzubak *et al*. 2006]. Triterpenoids have also been shown to possess anti-insect properties. Azadirachtin, a triterpenoid isolated from the Indian neem tree *Azadirachta indica* is a potent insect antifeedant with growth regulatory activity and reproductive effects [Gonalez *et al*. 2011].

The antifeedant properties of limonoids from *Melia azaderach* [China berry] and related plants have been reviewed [Nakatani, 1999]. The insect antifeedant and toxic effects of several triterpenes isolated from the latex of *Euphorbia* species have been described [Mazoir *et al*. 2008].

Betulin derivatives are effective agents against bollworm larvae [*Heliothis zea]* [Lugemwa *et al*. 1990] and Colarado potato beetle [*Leptinotarsa decemlineata*] [Huang *et al*. 1995]. The quassinoids are well known nortriterpenes with insecticidal properties which have been found in the Simarubaceae family [Almeida *et al*. 2007]. A pentacyclic triterpene, β-amyrin palmitate isolated from *Santalum album* acts as an insect growth inhibitor and exhibits chemosterilant properties [Shankaranarayana *et al. 1*980].

In this line of exploration of natural triterpenoids which could exhibit insect growth regulating activity, Betulinic acid [Fig 1], the bark extract of *Ziziphus jujuba* is choosen.

Betulinic acid is a widely distributed pentacyclic triterpene in the plant kingdom. Considerable amounts of Betulinic acid [up to 2.5%] is available in the outer bark of a variety of tree species that are valuable for timber purposes. White birch bark, *Betula alba* [which contains betulinic acid] has been used by Native Americans as a folk remedy. They used it in tea and other beverages to treat stomach and intestinal problems such as diarrhea and dysentery [Yogeeswari and Sriram, 2005].

Betulinic acid is isolated from various plants. It can be isolated from methanol, hexane and ethyl acetate extracts of stem bark of *Berlinia grandiflora* , stem barks of *Physocarpus intermedium* and *Tetracentron sinense*, chloroform extract of barks of *Syncarpa glomulifera*, dichloromethane extract of stem bark of Brazilian medicinal plant *Zizyphus joazeiro* etc [Yogeeswari and Sriram, 2005].

Betulinic acid is a very promising compound. This terpene has been extensively investigated for its pharmacological properties [Dzubak *et al.* 2006]. However, there is little information about its action on insect pests. Therefore an attempt has been made to study the growth regulating activity of Betulinic acid against the stored grain pest *Tribolium confusum.*

MATERIALS AND METHODS

Tribolium confusum were reared on mixed flour of wheat and jowar and maintained at 27 ± 1^{0}C temperature and 60 ± 5% relative humidity. Freshly moulted fifth instar, sixth instar larvae and zero hour pupae were treated topically on the abdominal region with 1µg of Betulinic acid dissolved in 1µl of acetone with the help of a Hamilton micro syringe. Thirty fifth instar, sixth instar larvae and zero hour pupae were treated each time and the experiments were replicated five times. Parallel controls treated with 1µl of acetone were maintained. After the treatment a suitable gap of time was given for the total absorption of the extract and they were transferred into the diet. The treated larvae and pupae were observed daily to note the changes.

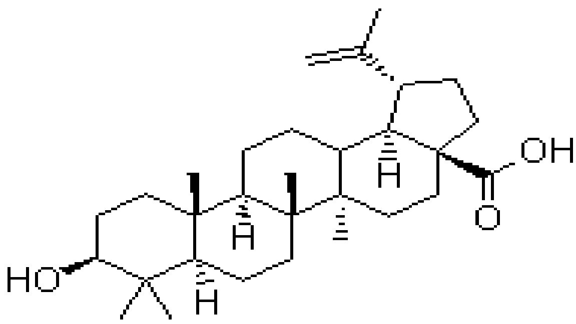

Fig1: Structure of Betulinic acid

RESULTS

Thirty fifth instar, sixth instar larvae and zero hour pupae of *Tribolium confusum* were treated topically with betulinic acid and the experiments were replicated five times. 1µg/µl of Betulinic acid resulted in moulting disruption and induced severe morphogenetic abnormalities in *Tribolium confusum*.

Effect of Betulinic acid on fifth instar larvae of *Tribolium confusum*

Among the treated fifth instar larvae few of them died during moulting, few remained as permanent larvae, some of them developed into larval-pupal intermediates, abnormal pupae and remaining treated fifth instar larvae developed into morphologically normal adults.

26.66% of the treated fifth instar larvae failed to moult and died after 2-3 days. 6.66% of the treated fifth instar larvae remained as "permanent larvae" and did not accomplish the pupal metamorphosis. 33.33% of the fifth instar treated larvae moulted into larval-pupal intermediates. Most of the intermediates produced exhibited pupal abdomen with larval thoracic legs. These larval-pupal intermediates suffered from ecdysial failure and larval exuviae remained attached to the posterior region of the body [Fig 6 A]. Such forms were inactive and their life cycle was terminated.

13.33% of the treated fifth instar larvae metamorphosed in to abnormal pupae exhibiting uneven tanning of the pupal case [Fig 6 B]. These abnormal pupae eclosed in to adults which failed to survive for a long time. 20% of the treated fifth instar larvae pupated normally and eclosed in to morphologically normal adults. [The percentages are depicted in Fig 2]

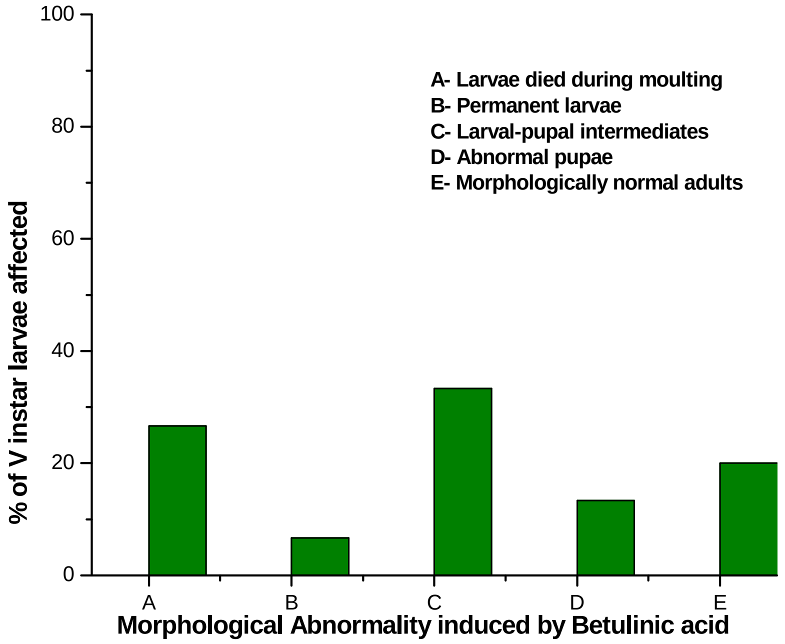

Fig 2. Morphogenetic effects of Betulinic acid against V instar larvae of *Tribolium confusum*

Effect of Betulinic acid on sixth instar larvae of *Tribolium confusum*

Some of the treated sixth instar larvae failed to pupate while few of them developed in to abnormal pupae, pupal-adult intermediates, abnormal adults and rest of them metamorphosed into morphologically normal adults.

30% of the treated sixth instar larvae failed to pupate and finally died. 23.33% of treated sixth instar larvae emerged into larval-pupal intermediates with exuvium attached to the body. These intermediate forms remained inactive and died after few days.10% of the treated sixth instar larvae metamorphosed in to abnormal pupae with exuvium attached to the abdomen [Fig 6 C]. These abnormal pupae failed to emerge into adults. 13.33% of the treated sixth instar larvae moulted in to pupal-adult intermediates. Adults developed within the pupal cuticle unable to shed the exuviae. These intermediates did not undergo subsequent developmental changes and ultimately died.

6.66% of the treated sixth instar larvae developed in to normal pupae which eclosed into abnormal adults. Most of these forms showed elongated antenna, fused transparent forewings and hindwings with no sign of sclerotization and the abdominal end covered with a cap of hard cuticle with no sign of external genitalia [Fig 6 D]. These forms were ruled out from further development and reproduction. 16.66% of treated sixth instar larvae pupated normally and morphologically normal adults eclosed from these pupae. [The percentages are depicted in Fig 3]

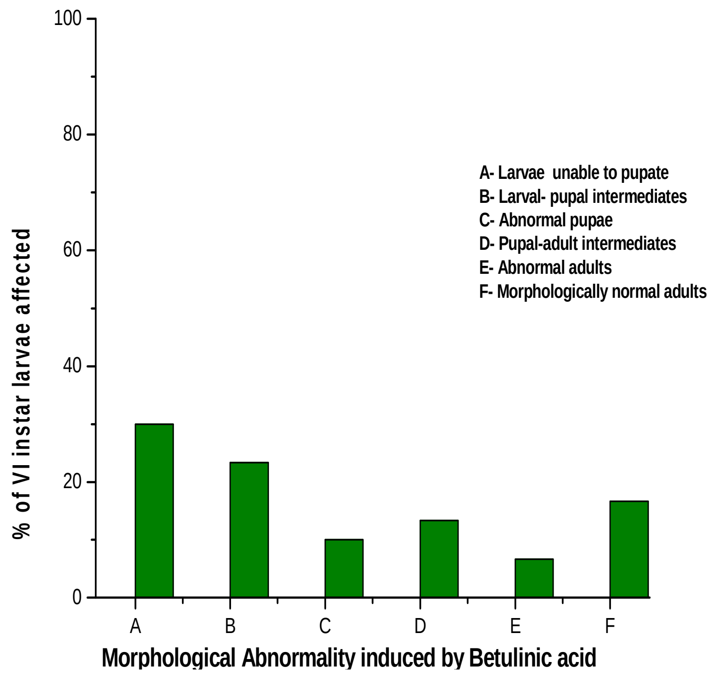

Fig 3. Morphogenetic effects of Betulinic acid against VI instar larvae of *Tribolium confusum*

Effect of Betulinic acid on zero hour pupae of *Tribolium confusum*
Few of the treated pupae died during adult eclosion, few of them developed into pupal-adult intermediates and abnormal adults and the remaining emerged as morphologically normal adults.

13.33% of the treated pupae failed to eclose in to adults. 33.33% of the treated pupae moulted in to pupal-adult intermediates. They were unable to extricate from the pupal case exhibiting ecdysial failure. These forms were ruled out from further development. 30% of the treated pupae developed in to abnormal adults with exuviae seen attached to appendages and posterior region which prevented the insects from flying, defaecation and mating [Fig 6 E, F]. 23.33% of the treated pupae metamorphosed in to morphologically normal adults. [The percentages are depicted in Fig 4]

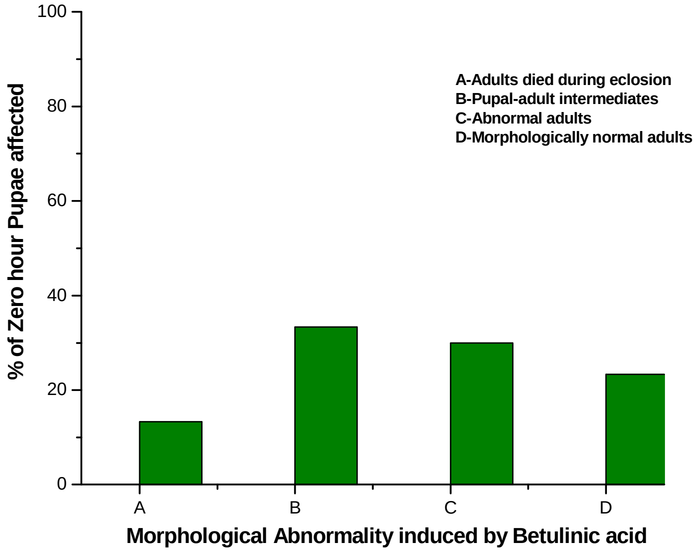

Fig 4. Morphogenetic effects of Betulinic acid against Zero hour pupae of *Tribolium confusum*

Betulinic acid induced a decrease in the percentage of adult emergence compared to the controls. Only 19.99 ± 3.335 adult emergence was observed with Betulinic acid while 100% adult emergence was seen in controls [Fig 5].

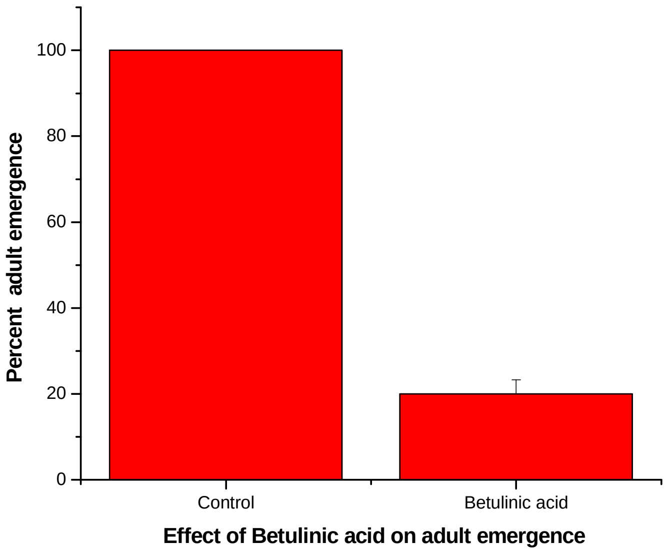

Fig 5. Effect of Betulinic acid on adult emergence of *Tribolium confusum*.

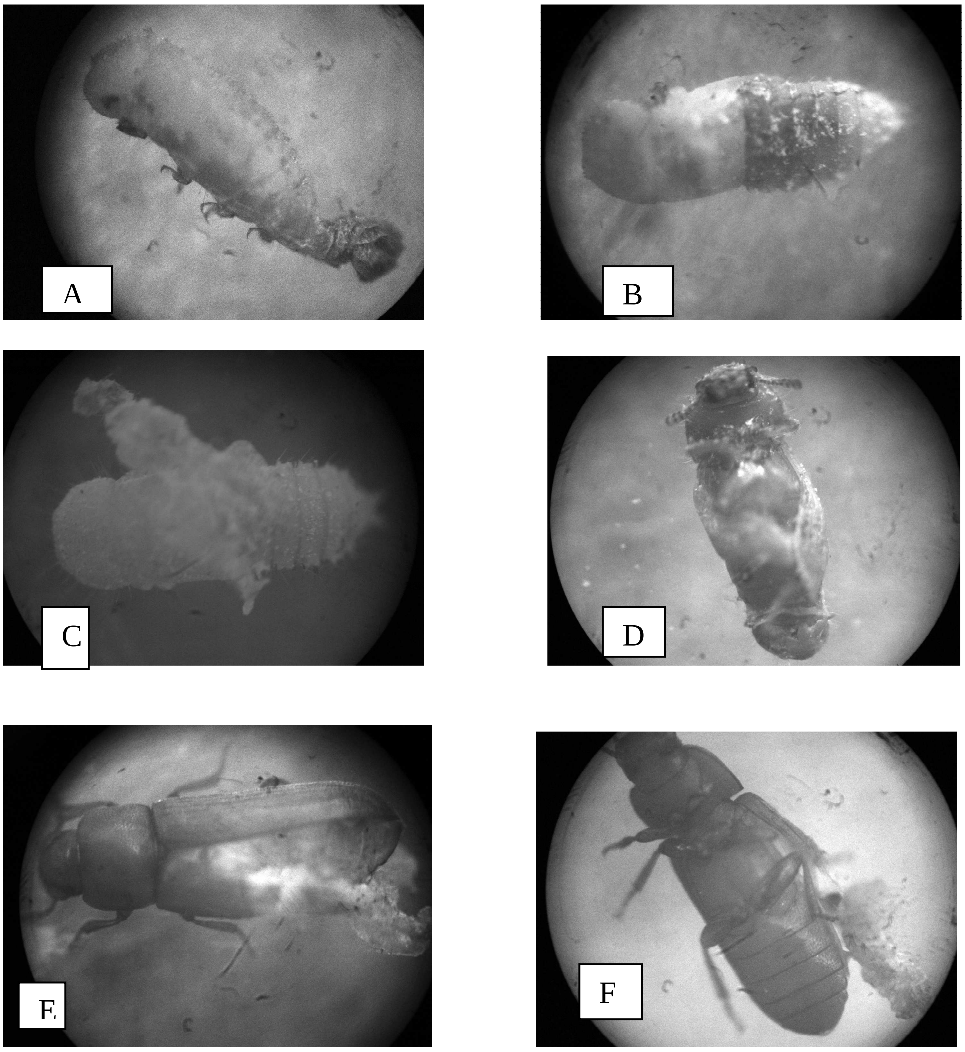

Fig 6

A. Larval-pupal intermediate with larval thoracic legs and exuvium attached to the posterior end
B. Pupa exhibiting uneven tanning of the pupal case
C. Pupa with exuvium attached to the abdomen
D. Abnormal adult with elongated antenna and fused forewings and hind wings with abdominal end covered with a cap of hard cuticle
E. Adult with exuvium attached to the posterior region
F. Adult with exuvium attached to the appendages

DISCUSSION

The present study suggests that topical application of Betulinic acid prevented normal development and metamorphosis of *Tribolium confusum,* which was manifested at different stages of the life cycle.

Most of the treated fifth instar larvae of *Tribolium confusum* died after the moult was prolonged for several days. This may be due to an imbalance in the hormone titers at critical times of moulting [Retnakaran *et al.*1985]. Similar observation was reported by Martinez and Embden [2001] in *Spodoptera littoralis* [Boisduval] treated with azadirachtin.

Gandhi *et al*. [2010] reported that the extracts of plants like *Annona squamosa* [L.], *Lantana camara, Cleodendrum inerme, Cassia fistula, Azadirachta indica* and *Calotrophis procera* interfere with moulting process.

Some of the treated fifth instar larvae of *Tribolium confusum* larvae remained as permanent larvae which showed prolonged larval periods. The prolongation of larval duration may be due to the persistence of juvenile hormone in the haemolymph where it is only in the absence of juvenile hormone that ecdysone could be activated and lead to the formation of the next stage [Kuwano *et al*. 2008].

Most of the treated sixth instar larvae of *Tribolium confusum* failed to pupate. It is likely that pupation in treated larvae is inhibited by disturbance in ecdysteroid regulation shortly before ecdysis as observed in *Helicoverpa armigera* [Nabawy Elkattan *et al*. 2001].

Inhibition of metamorphosis was observed to varying degrees. Some of the treated fifth instar larvae of *Tribolium confusum* moulted in to larval-pupal intermediates. Most of these intermediates produced exhibited pupal abdomen with larval thoracic legs. These suffered from ecdysial failure and larval exuviae remained attached to the posterior region of the body preventing their further development. This suggests that betulinic acid might act as an insect growth inhibitor, where Wright [1970] indicate that such malformation [larval-pupal intermediate] is a true juvenile hormone effect.

Formation of larval-pupal intermediates was also observed in the larvae of *Synthesiomyia nudiseta* treated with botanical volatile oils [Abdel Fattah A. Khalaf *et al.* 2009].

Some of the treated fifth instar and sixth instar larvae of *Tribolium confusum* metamorphosed in to abnormal pupae. The abnormal pupae that managed to eclose into adults failed to survive for a long time. Few of the treated sixth instar larvae emerged into pupal-adult intermediates. Adults developed within the pupal cuticle unable to shed the exuviae. These intermediates did not undergo subsequent developmental changes and ultimately died. Similar observations were also noticed in *Dysdercus similis* treated with methanol leaf extract of *Chrysanthemum indicum* [Kaur et al. 1989].

The inability of the insects to extricate from the old exuvium during ecdysis, is one of the most common effects, associated with juvenile hormone action on morphogenesis. Such inhibition is of considerable practical significance since most of the insects which fail to ecdyse, are ruled out from further reproduction.

Deformities and death in larval, larval-pupal intermediate, pupal and pupal-adult intermediate stages may be due to change in the ecdysteroid titre as demonstrated in coffee bug treated with methanol extract of neem leaves [Prathiba V. Deshmuke *et al*. 2011]. Similar morphogenetic effects of Azadirachtin rich fractions against *Spodoptera litura* were observed by Nelson and Venugopal [2006].

Some of the treated resultant pupae of *Tribolium confusum* exhibited uneven tanning of the pupal case. According to Hori *et al.* [1984] this may be due to low levels of ecdysone.

Those treated sixth instar larvae of *Tribolium confusum* that metamorphosed into pupae eclosed into abnormal adults. Most of these forms showed elongated antenna, fused transparent forewings and hind wings with no sign of sclerotization and the abdominal end covered with a cap of hard cuticle with no sign of external genitalia. These forms were ruled out from further development and reproduction. These observations signifies a derangement in endocrine mechanisms. Similar results were recorded by Shaheen and Osmani [1980] on castor semi-looper moth.

The gradual changes that occur in the successive stages of insects, especially the more striking changes in metamorphosis are likewise controlled by a delicate balance in timing and concentrations of the secretions from the corpus allatum and prothoracic gland. Application of Betulinic acid, probably disturbed the delicate balance of timing and concentration of the hormones in the intact insect, resulting in the formation of abnormal, non-viable forms.

Most of the trerated pupae of *Tribolium confusum* moulted in to pupal-adult intermediates which failed to extricate from the pupal case while few of the pupae eclosed in to abnormal adults with exuviae seen attached to appendages and posterior region preventing the insects from flying, defaecation and mating. Several adult deformities were also observed in *Schistocerca gregaria* after treatment with an essential oil from *ageratum conyzoides* [Pari *et al.* 2000].
The adult malformation of *Tribolium confusum* can be attributed to the intervening of Betulinic acid with the hormonally controlled program of morphogenesis. This may be due to the modification of ecdysteroid titer, which in

turn leads to changes in lysosome enzyme activity causing overt morphological abnormalities as also reported by Samira A.Aly *et al.* [2010] working with different extracts of *Fagonia bruguieri* on *Schistocerca gregaria.*

A very few of the treated larvae and pupae metamorphosed into morphologically normal adults. Betulinic acid exhibited moult inhibition, affected larval growth and development, deranged pupal and adult morphogenesis and inhibited adult emergence. Inhibition of adult emergence was also observed in *Callasobruchus maculatus* with various plant products [Shifa vanamathi *et al.* 2010].

Application of Betulinic acid to larvae and pupae prevented subsequent development to adult stage, thus reducing the reproductive potential of the population. The disruption of reproductive capability could lead to substantial population decline overtime.

CONCLUSION

Our results infer that Betulinic acid, a terpenoid extracted from the bark of *Ziziphus jujuba* which is eco-friendly and cost effective shows effective insect growth regulating activity and exhibits great promise in suppressing the population of the stored grain pest *Tribolium confusum.*

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