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Research Protocol

Ayurveda Section

Pharmaceutico-analytical Study of *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu* and their Comparative In-vivo Evaluation of Anti-osteoporotic Activity: A Research Protocol of Experimental Study

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ABSTRACT

Introduction: Osteoporosis is a progressive bone disease characterised by a decrease in bone mass and density. It is a major public health problem and a significant risk factor for fractures, especially among elderly women. Additionally, long-term Glucocorticoid (GC) therapy is associated with a significant reduction in bone density and is one of the most common causes of secondary osteoporosis.

Need of the study: Conventional medical management of osteoporosis typically involves calcium supplementation, Hormone Replacement Therapy (HRT), and the use of drugs such as bisphosphonates, which reduce osteoclast activity and, atleast in the short term, increase bone density. However, these interventions slow overall bone turnover. The present study aims to investigate the effectiveness of the Ayurvedic medicine *Lakshadi Guggulu* and its modified form, *Pravalyukta Lakshadi Guggulu*, in managing osteoporosis. According to Ayurvedic classics, *Lakshadi Guggulu* is a highly effective calcium formulation for correcting bone metabolic disorders, facilitating fracture repair, treating joint dislocations, and providing analgesic and anti-inflammatory effects.

Aim: To conduct a pharmaceutico-analytical study of *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu*, and to evaluate their comparative anti-osteoporotic activity in vivo.

Materials and Methods: The present experimental study will be conducted from June 2025 to November 2025 in the animal house at Datta Meghe College of Pharmacy (DMCP), Datta Meghe Institute of Higher Education and Research (DMIHER) Deemed University (DU) Wardha, Maharashtra, India. Raw materials will be collected from the local market and verified and authenticated by the Taxonomist/*Dravyaguna* Department. The medicines will be prepared, standardised, and analysed at Mahatma Gandhi Ayurved College Hospital and Research Centre (MGACH and RC) as per the Ayurvedic Pharmacopoeia of India (API). Analytical studies will be conducted for both *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu*. The animal study will adhere to Organisation for Economic Co-operation and Development (OECD) guidelines 423 and include a total of 78 female Wistar rats: 24 rats will be used for the acute toxicity study, and the remaining 54 rats will be divided into nine groups for the efficacy study. General clinical observations, biochemical and haematological parameters, and histopathological examinations will be conducted. Data will be statistically analysed using the Student's t-test and Analysis of Variance (ANOVA) as appropriate. A p-value <0.05 will be considered statistically significant.

Keywords: Bone density, Glucocorticoid induced osteoporosis, Hormone replacement therapy, Osteoclast

INTRODUCTION

Osteoporosis, a degenerative bone disease characterised by reduced bone mass and density, is a major public health concern and a leading cause of fractures in older adults, particularly women. Postmenopausal (primary type 1) osteoporosis commonly affects women after menopause, with the World Health Organisation (WHO) estimating that 30% of women are affected. In India, approximately 20% of women over 50 are projected to have osteoporosis [1]. Senile (primary type 2) osteoporosis occurs in both men and women over 75 years, whereas secondary osteoporosis results from chronic illnesses or prolonged steroid use [2].

Glucocorticoids (GCs) are widely used to treat chronic inflammatory and autoimmune conditions, like asthma, Crohn's disease, and rheumatic disorders [3]. However, long-term use significantly reduces bone mineral density, making GC-induced osteoporosis (GIOP) the leading cause of secondary osteoporosis in individuals under 50 years [4]. Despite its severity, GIOP is often underdiagnosed and undertreated, representing a major concern for patients requiring prolonged GC therapy.

Ayurveda, the science of life, is grounded in timeless principles. *Asthi Dhatu Kshaya* refers to the progressive loss of bone quality, initially affecting teeth, hair, and nails, comparable to osteopenia. As the condition worsens, it leads to *Asthi-soushirya* (bone porosity), akin to osteoporosis, eventually impacting the marrow (*Majjadhatu*). *Kshaya* denotes a qualitative or quantitative decline of tissue, thus *Asthikshaya* implies a reduction in bone tissue [5].

Asthi possesses properties such as heaviness (*guru*), roughness (*khara*), hardness (*kathina*), bulk (*sthula*), stability (*sthira*), and form (*murtimad*) [6]. It supports body structure (*deha dharana*), nourishes bone marrow (*Majjapushti*), and serves as the seat of *Vata* [7]. Although specific causes of *Asthikshaya* are not separately listed, Charaka mentions general aetiological factors, including *Vata*-aggravating habits like excessive exercise, dry and irregular diets, emotional stress, sleep deprivation, ageing, and seasonal influences [8]. These factors lead to *Ama* formation and *Vata* vitiation through *Marga-avarana* (obstruction) or *Dhatukshaya* (tissue depletion) [9]. Consequently, there is dryness of the *srotas*, impaired *Agni* (digestive/metabolic fire), and poor Asthi nourishment. As *Asthi*

attributes like *guru*, *kathina*, and *sthira* diminish, bone porosity (*Asthi-soushirya*) develops.

The standard medical approach to osteoporosis generally involves calcium supplementation, Hormone Replacement Therapy (HRT), and medications such as bisphosphonates, which reduce osteoclast activity and help increase bone density in the short term [10,11]. While many people are aware of the potential risks of HRT, fewer are aware that bisphosphonates can also cause side effects lik headaches, constipation, diarrhoea, stomach pain, acid reflux, ulcers, muscle and joint pain, cramps, dizziness, and fatigue. These medications initially improve bone density by slowing bone resorption, but over the long term, they reduce the bone turnover rate [12]. As a result, old or damaged bone may accumulate, potentially increasing bone fragility over time.

Given these concerns, there is growing interest in exploring natural remedies for osteoporosis due to their generally safer profiles and multiple beneficial activities. The Indian System of Medicine (ISM), particularly Ayurveda, offers a comprehensive *materia medica* and serves as a valuable alternative for chronic conditions often associated with side effects from prolonged allopathic treatment [13]. With over 2,500 years of documented practice, Ayurveda views the body holistically and employs individualised therapeutic strategies.

Management of *Asthikshaya* includes *Nidanaparivarjana* (removal of causes), *Shodhana* (purification), *Shamana* (palliation), *Rasayana* (rejuvenation), and *Pathya-Apathya* (diet and lifestyle). The core approach is *Samprapti Vighatana*—disruption of pathogenesis. Therefore, treatment of *Asthikshaya* should consider both factors responsible for *Vata* aggravation, namely *Margavaran* (channel obstruction) and *Dhatu Kshaya* (tissue depletion) [14].

Margavarana is managed using *Srotoshodhana* herbs with *Katu* and *Tikta rasa*, whereas *Dhatu Kshaya* is treated with *Vata-shamana*, *Tarpana*, and *Brimhana* therapies employing herbs with *Guru*, *Snigdha guna*, *Madhura Vipaka*, and *Ushna Virya*.

According to Bhaishajya Ratnavali, *Lakshadi Guggulu* is predominantly used in *Asthigatavikara* (bone disorders), such as *Bhagna* (fracture) and Asthichyut (dislocation) [15]. Yogaratnakar describes its use for immediate fracture repair, joint dislocations, and as an analgesic and anti-inflammatory agent [16].

Objectives:

- To prepare and conduct an analytical study of *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu*.
- To evaluate and compare the acute oral toxicity and anti-osteoporosis activity of *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu* in GC-induced osteoporosis.

Null Hypothesis (H0): There is no significant difference in the pharmaceutico-analytical characteristics or anti-osteoporosis activity between *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu* in glucocorticoid-induced osteoporosis.

Alternate Hypothesis (H1): *Pravalyukta Lakshadi Guggulu* exhibits significantly different pharmaceutico-analytical characteristics and superior anti-osteoporosis activity compared to *Lakshadi Guggulu* in glucocorticoid-induced osteoporosis.

REVIEW OF LITERATURE

Pravalyukta Lakshadi Guggulu (PLG) is a modified form of *Lakshadi Guggulu*, containing its ingredients along with *Praval*. *Praval* is a natural, calcium-rich substance derived from oyster shells and commonly used to treat calcium-deficiency-related bone metabolic disorders. When properly processed using Ayurvedic methods such as *Bhavana*, *Shodhana*, and *Marana*, *Praval* is readily absorbed through the intestines [17].

Other commonly used ingredients include *Shodhit Laksha* (processed *Laccifer lacca*), *Asthishrunkhala* (*Cissus quadrangularis*), and *Arjuna* (*Terminalia arjuna*), which help accelerate fracture healing [18-20]. *Shodhit Guggulu* (processed *Commiphora wightii*) is known for its anti-resorptive properties [21], while *Ashwagandha* (*Withania somnifera*) is a prime Rasayana (anti-ageing) herb. Treatment with Withania somnifera markedly prevents increases in serum ALP, calcium excretion, and metaphyseal bone loss in OVX rats, making it a potential agent for osteoporosis management [22].

Limited research exists on the analytical study and anti-osteoporosis activity of *Lakshadi Guggulu* in-vivo. Namana H et al., conducted a comparative analytical study of *Lakshadi Guggulu*, testing prepared samples against market tablets and revealing significant variation in quality control parameters among the four marketed products [23]. Singh SK et al., evaluated *Lakshadi Guggulu*, Tiktadi oil Matrabasti, and Tiktadi Ksheerbasti in *Asthikshaya* relative to osteoporosis. The study concluded that combined therapy with Basti and *Lakshadi Guggulu* tablets was most effective in managing *Asthikshaya*/osteoporosis [24].

Gupta AK et al., examined the effects of Majjabasti (therapeutic enema) and Asthishrunkhala in osteoporosis management (Asthi-Majjakshaya). The study observed that this combination was effective in treating osteoporosis and improved patients' overall health [25]. Dudhamal T studied the efficacy of *Lakshadi Plaster* and *Lakshadi Guggulu* in *Bhagna* (stable collar's fracture), finding that the plaster achieved immobilisation comparable to Plaster of Paris without complications, while internal *Lakshadi Guggulu* promoted early bone healing without additional calcium or vitamin supplementation [26].

However, there remains a lack of standardised experimental evidence regarding the positive effects of *Lakshadi Guggulu* and its modified form, *Pravalyukta Lakshadi Guggulu*, on bone health. Therefore, the present pharmaceutico-analytical study will be conducted to evaluate these preparations and compare their anti-osteoporosis activity in-vivo.

MATERIALS AND METHODS

The experimental study will be conducted from June 2025 to November 2025 in the animal house at DMCP, DMIHER (DU), Wardha, Maharashtra, India. The research has been approved by the Institutional Animal Ethics Committee (IAEC) of Datta Meghe Institute of Higher Education and Research under reference number DMIHER/IAEC/2024-25/25.

The animal study will adhere to OECD guidelines 423 [27] and will include a total of 78 female Wistar rats: 24 rats will be used for the acute toxicity study, and the remaining 54 rats will be divided into nine groups for the efficacy study.

Analytical studies will be conducted at Cotex Laxmi Health Care Pvt. Ltd. and MGACH and RC, Salod (H), Wardha. Additional analyses and experiments will be performed at a nationally recognised laboratory or research institute listed in the DMIHER (DU) profile.

Inclusion criteria:

- Only female Wistar rats.
- Average age of the rats will be approximately three months.
- Weight between 180-220 g.

Exclusion criteria:

- Pregnant or diseased female rats.
- Rats currently involved in other experimental trials.

Study Procedure

The procurement of raw materials will be done from Mankarnika Ayurvedic Store, Pune. The taxonomist or the *Dravyaguna* Department will verify and authenticate the raw pharmaceuticals. Raw pharmaceuticals will be standardised by API or analytical laboratories such as MGACH and RC, while additional analysis will be performed at the Central Research Lab of Jawaharlal Nehru Medical College.

The formulation will be prepared according to the Standard Operating Procedures (SOPs) outlined in the Ayurvedic Pharmacopoeia of India. Quality raw materials will be sourced and verified. Each ingredient will be washed with clean water and thoroughly dried. Dried ingredients 1-5 will be individually pounded into a fine powder, sifted through an 85-mesh sieve, weighed accurately, and mixed to create a homogeneous blend [Table/Fig-1] [15].

S. No.	Name of ingredient	Botanical name	Part used	Quantity
1	*Laksha*	*Lacciferlacca*	Exudate	1p
2	*Asthishrunkhala*	*Cissus quadrangularis*	Stem	1p
3	*Arjun*	*Terminalia arjuna*	Stem bark	1p
4	*Ashwagandha*	*Withaniasomnifera*	Root	1p
5	*Nagabala*	*Greviahirsuta*	Root	1p
6	*Guggulu*	*Commiphora myrrha*	Resin	5p

[Table/Fig-1]: Ingredients of *Lakshadi Guggulu* with botanical name, used part and quantity [15].

Shuddha Guggulu will be mixed with an equal volume of water, transferred to an iron vessel, and gently boiled until thickened. The powder mixture will then be added gradually while stirring, and trituration will be performed in a *Khalwa Yantra* with added ghee until a uniform mass is formed. This mass will be shaped into round pills and dried in a tray dryer at a temperature not exceeding 60°C for 10-12 hours. Once dried, the pills will be packed in airtight containers and stored in a cool, dry place away from light and moisture.

Justification of *Praval Bhasma* dose: According to Rasa Tarangini (23/142), the dose of *Praval Bhasma* is ½-2 Ratti (65-250 mg) per day [28], and the dose of *Lakshadi Guggulu* is 500 mg-1 g per day [15]. Considering the same dose for *Pravalyukta Lakshadi Guggulu*, each 500 mg tablet will contain 65 mg of *Praval Bhasma*.

Preparation of *Praval Bhasma*: *Praval* will be kept in muslin cloth and tied into a *pottali*. This *pottali* will be suspended in a pot containing *Sarjjiksharjala* (Sajjikshar + water) using a rod and heated continuously for three hours. The *pottali* will then be removed, and the *Praval* will be washed and dried [28].

The dried *Shuddha Praval* will be powdered, mixed with *Ghrutkumariswarasa*, and triturated. *Chakrika* will be prepared from this mixture, dried in the shade, and placed in a *Sharav*. The *Sharav Samputa* will be made using *matkapad* and *Multani mitti*, then dried. This *Sharav Samputa* will be subjected to *Gajaputa*. After cooling, the process will be repeated once more. Two *Gajaputa* cycles are required to form *Praval Bhasma* [28].

Method of preparation of *Pravalyukta Lakshadi Guggulu:* All ingredients will be selected for quality and authenticated through standard identification procedures. Each ingredient will be washed individually with clean, soft water and thoroughly dried [Table/Fig-2]. Upon complete drying, raw ingredients 1-5 will be pounded separately into fine powders. These powders will be sieved individually through mesh no. 85 to ensure uniform particle size. Each powdered ingredient will be weighed as per the required quantity and mixed thoroughly to form a homogeneous blend.

S. No.	Name of ingredient	Botanical Name	Part used	Quantity
1	*Laksha*	*Lacciferlacca*	Exudate	100 gms
2	*Asthishrunkhala*	*Cissus quadrangularis*	Stem	100 gms
3	*Arjun*	*Terminalia arjuna*	Stem bark	100 gms
4	*Ashwagandha*	*Withaniasomnifera*	Root	100 gms
5	*Nagabala*	*Sidaveronicafillia*	Root	100 gms
6	*Praval Bhasma*	*Coral*	Incinerated Ash	125 gms
7	*Guggulu*	*Commiphora myrrha*	Resin	500 gms

[Table/Fig-2]: Ingredients of *Pravalyukta Lakshadi Guggulu* with its botanical name, used part and quantity.

The required quantity of *Shuddha Guggulu* will be mixed with an equal volume of water and transferred to an iron vessel. The mixture will be gently boiled until a thick, viscous consistency is achieved. The previously prepared homogeneous powder mixture will then be added gradually with continuous stirring.

Next, the resulting mixture will be transferred to a *Khalwa Yantra* (traditional mortar and pestle apparatus). The specified quantity of *Praval Bhasma* will be added, and the entire mixture will be triturated thoroughly. To facilitate the trituration process, a small amount of castor oil or ghee will be incorporated. Trituration will continue until a semi-solid, homogeneous mass is obtained.

This mass will then be rolled into uniformly round pills using the palm. The prepared pills will be placed in a tray dryer and dried at a temperature not exceeding 60°C for 10-12 hours. Once completely dried, the pills will be packed in airtight containers and stored in a cool, dry place, protected from light and moisture, to maintain their stability and therapeutic efficacy.

An animal study (acute oral toxicity and efficacy study) will be carried out according to OECD guidelines 423 [27]. Animals will be obtained from a certified source and housed in polypropylene cages under standard conditions of temperature (23±2°C) and relative humidity (55±10%), with a 12-hour light-dark cycle. Food and drinking water will be supplied ad libitum.

Total 24 rats will be used for the acute toxicity study [Table/Fig-3], and the remaining 54 rats will be divided into nine groups for the efficacy study [Table/Fig-4].

S. No.	Groups	No of animal required	Treatment	Dose	Route
01	Lower toxicity dose group	06	LG	2 gms/kg body weight	Oral
02	Higher toxicity dose group	06	LG	5 gms/kg body weight	Oral
03	Lower toxicity dose group	06	PLG	2 gms/kg body weight	Oral
04	Higher toxicity dose group	06	PLG	5 gms/kg body weight	Oral

[Table/Fig-3]: Grouping and treatment plan for acute oral toxicity study of LG and PLG extracts in rodents.

S. No.	Groups	No of animals required	Treatment	Dose	Duration	Route
01	Control group 1 (toxic control) Positive osteoporosis in rats	06		*		Oral
02	Control group 2 (normal control group) No osteoporosis in rats	06	Saline water (Vehicle)	*	12 weeks	Oral
03	Standard group	06	Alendronate	35/mg/kg/day	12 weeks	Oral
04	Test group (high dose group)	06	LG	*	12 weeks	Oral
05	Test group (medium dose group)	06	LG	*	12 weeks	Oral
06	Test group (Low dose group)	06	LG	*	12 weeks	Oral
07	Test group (high dose group)	06	PLG		12 weeks	Oral
08	Test group (medium dose group)	06	PLG		12 weeks	Oral
09	Test group (Low dose group)	06	PLG		12 weeks	Oral

[Table/Fig-4]: Experimental group design for evaluating the anti-osteoporotic activity of LG and PLG in rats.

A total of 24 animals will be divided into four groups (n=6 per group). The study will assess the acute toxicity of *Lakshadi Guggulu* (LG) and *Pravalyukta Lakshadi Guggulu* (PLG) administered orally at two different dose levels: 2 g/kg (lower dose) and 5 g/kg (higher dose) body weight. All treatments will be given orally. Animals will be monitored for signs of toxicity over a 14-day period post-administration.

Procedure for acute toxicity study: Female Wistar rats will be fasted for 16-18 hours prior to dosing. Food will be withheld for 3-4 hours post-dosing, but drinking water will be provided ad libitum. The starting dose will be 2 g/kg body weight p.o., and the highest dose will be 5 g/kg body weight p.o. Mortality will be checked at both dose levels. The initial dose should correspond to the level at which animals are most likely to exhibit toxic effects. The time interval between doses will be determined based on the onset, duration, and severity of toxic signs.

Animals will be observed for a total of 72 hours, starting with continuous monitoring during the first 30 minutes, periodic observations over the first 24 hours, and specific attention during the first four hours. Daily observations will continue thereafter. Parameters to be monitored include:

- Toxic signs, severity, onset, progression, and reversibility
- Mortality, if any
- Changes in eyes, mucous membranes, skin, and fur
- Alterations in circulatory, respiratory, autonomic, and central nervous systems
- Somatomotor activity and behavioural patterns
- Tremors, convulsions, salivation, diarrhoea, lethargy, sleep, or coma
- Further observations will be made if any signs of toxicity are detected.

Efficacy study for osteoporosis: Glucocorticoid-induced Osteoporosis (GIOP) will be induced by intramuscular injection of dexamethasone at 25 mg/kg twice per week for six weeks. After the development of osteoporosis, LG and PLG will be administered orally for 12 weeks. The doses of LG and PLG will be selected based on the results of the acute toxicity study. At the beginning and end of the treatment, animals will be examined for Bone Mineral Density (BMD) and serum bone Alkaline Phosphatase (ALP). Bone loss can be measured using Dual Energy X-ray Absorptiometry (DXA), micro-computed tomography, or biochemical parameters to characterise the GIOP bone phenotype. Osteoporosis will be induced in all groups except the normal control group [Table/Fig-4]. Treatment will be administered orally for a period of 12 weeks.

Outcomes

Primary outcome: Analytical study of *Lakshadi Guggulu* and *Pravalyukta Lakshadi Guggulu*

Organoleptic characteristics [29]:

a. Sparsha (Touch);

b. Rupa (Appearance and colour);

c. Rasa (Taste);

d. Gandha (Odour).

Physicochemical parameters:

a. **Loss on drying:** A total of 10 grams of the drug will be uniformly spread in a thin layer within a shallow Petri dish. The dish will be subjected to controlled heating at 105°C, followed by cooling in a desiccator before weighing. This procedure will be repeated until two consecutive weight measurements remain stable. The percentage of weight loss will then be calculated relative to the initial weight [29].

b. **Ash value, w/w:** Two grams of each sample will be accurately weighed and placed into silica crucibles. The samples will be evenly spread, incinerated, allowed to cool, and weighed. The total ash value will be obtained by subtracting the weight of the empty crucible from the weight of the crucible containing the incinerated sample [29].

c. **Acid-insoluble ash:** Residues from the total ash determination will be boiled in hydrochloric acid, rinsed with hot water, placed in a crucible, dried, and weighed. The difference between the weight of the crucible containing the incinerated sample and the empty crucible will yield the acid-insoluble ash value [29].

d. **Alcohol-soluble extractive:** The percentage of alcohol-soluble matter will be determined using the same method as described for water-soluble extractives, substituting alcohol for water [29].

e. **Water- soluble extractive:** Five grams of the drug will be mixed with 100 mL of distilled water and alcohol in a glass-stoppered conical flask. The mixture will be shaken at regular intervals for six hours and left to stand for 18 hours. After filtration, 25 mL of the solution will be evaporated to dryness in a water bath. The residue will then be dried at 105°C for six hours, cooled in a desiccator for 30 minutes, and weighed. The percentage of water-soluble matter will be calculated relative to the amount of air-dried drug [29].

f. **pH (1% aqueous solution):** The pH will be measured using a digital pH meter with a combined electrode. The instrument will be calibrated with buffer solutions of pH 4.0, 7.0, and 9.20 prior to measurement [29].

g. **HPLC/LCMS:** High Performance Liquid Chromatography (HPLC) will be used to separate, identify, and quantify components in the mixture. Liquid Chromatography-Mass Spectrometry (LC-MS) will combine HPLC separation with mass spectrometric analysis, providing both identification and quantification based on molecular mass [29].

H **XRD/ FTIR:** Fourier Transform Infrared Spectroscopy (FTIR) will be used to identify chemical bonds in *Praval Bhasma*, while X-ray Diffraction (XRD) will determine the crystalline structure of the molecules [30].

General clinical observations: Observations should be made at least once daily at the same time, considering the peak period of anticipated effects after dosing:

a General signs: Home cage activity, convulsions, biting behaviour.

b Physical observations: Eye prominence, hair coat condition, lacrimation, salivation, faecal excretion, urine output, locomotor activity, tremors, and respiration rate, to be recorded at least twice a week.

c Body weight: Regular recording.

d Food intake: Monitored consistently.

Biochemical parameters: Serum bone alkaline phosphatase and serum calcium levels.

Haematological examination: Haemoglobin, White Blood Cells (WBC), Red Blood Cell (RBC), platelet count, haematocrit, Erythrocyte Sedimentation Rate (ESR). All examinations will be performed at 0-, 30-, 60-, and 90-day intervals.

Histopathological examination: After six weeks, animals will be sacrificed, and femur bones will be prepared for histological evaluation, as well as for measurements of bone elasticity and hardness.

STATISTICAL ANALYSIS

Data obtained from the analytical and in-vivo experimental studies will be presented in tables and graphs. All values will be expressed as mean±Standard Error of the Mean (SEM). Statistical analysis will be performed using Student's t-test and ANOVA as required. Additional statistical tests may be applied as appropriate. A p-value <0.05 will be considered statistically significant.

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