

## Bioprospecting of *Adhatoda vasica* for Identification of Novel Compounds using Chromatographic Methods and Screening for Anti-diabetic and Antioxidant Activity

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### ABSTRACT

This investigation column eluted fractions of leaf *Adhatoda vasica* of was assessed for its phytochemical screening, column chromatography, thin layer chromatographic studies, protease activity, anti-inflammatory activity, antidiabetic activity and antioxidant activity. Phytochemical screening reflects the presence of alkaloid, flavonoids, coumarins, terpenoids, steroids, emodin's, Quinone's. Column chromatography method was used for purification of bioactive compounds. Thin layer chromatographic study was carried out by using various solvent system of different type of polarity of *n*- butanol, acetic acid and acetone. TLC profiling shows pure band at 254nm and 366 nm. The strong "proteolytic activity" also pointed out in purified fraction of eluted fraction. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation fraction 3, showing highest activity 75% followed by fraction 5 (62.73%), membrane stabilization assay fraction 6 (80.23%) followed by fraction 3 (64.65%) and proteinase inhibitory activity of fraction 5(88%) followed by fraction 7 (87.68%) at concentration 500 µg/ml. Aspirin (90.87%) was used as standard drug for the study of anti-inflammatory activity. *In vitro* antidiabetic activity was performed using Alfa amylase inhibition assay. Highest activity was showed in fraction 4 (79.05 %) and fraction 5 (77.05 %) at concentration 500 µg/ml. Antioxidant activity was performed by reducing power assay fraction number 2 has higher absorbance 1.04 at 500µg/ml followed by reducing power of column eluted fraction was compared with ascorbic acid as standard showing higher absorbance 0.93 at 500µg/ml.

**Keywords-** *Adhatoda vasica*, phytochemical screening, column chromatography, thin layer chromatographic studies, antidiabetic activity and antioxidant activity.

## I. INTRODUCTION

The increase in prevalence of multiple drug resistance has shown the development of new synthetic antimicrobial, antioxidant, anti-diabetic and anti-inflammatory drugs (Hamidpour, R., et al.2017); moreover the new drug is necessary to search for new antimicrobial, antioxidative, antidiabetic and anti-

inflammatory sources from alternative sources. Bioactive compounds from medicinal plant showing pharmacological activities have the potential of filling this need because their structure are different from those of the most studied plants, while those with more action may likely differ (Vaou, Natalia, et al 2021). (Commisso, Mauro, et al. 2021). *Adathoda vasica* is indigenous to India, where it is found in sub-Himalayan

track up to an altitude of 1000 m and in Maharashtra especially in Konkan region. Besides India (Sobia, H. et al. 2018), it is found in Myanmar, Sri Lanka and Malaya. The leaves, flowers, fruits and roots are extensively used for treating Cold, Cough, Whooping cough, Chronic bronchitis and Asthma (Gangwar, A. K., & Ghosh, A. K. 2014). It is also used considerable interest for its beneficial effects in Malaria, Dysentery, Diarrhea, Antimicrobial, Anthelmintic and Antiperiodic (Shamsuddin, Tahmida, et al. 2021). Owing to their immense importance and varied bioactivities exhibited by *Adhatoda vasica*, efforts have been made from time to time to generate libraries of isolated compounds and screen them for potential biological activities (Masondo, Nqobile A., et al. 2019). Chemical constituents of *Adhatoda vasica* were isolated successfully with higher yield. The structures of isolated compounds were confirmed by the use of spectral data UV, Mass and HPLC. The extract of *Adhatoda vasica* was evaluated for Antidiabetic activity. (Bhanukiran, Kancharla, et al. 2023). The typical bioactive compounds in plants are produced as secondary metabolites. Free radicals which have one or more unpaired electrons (superoxide, hydroxyl, peroxyl) are produced in normal or pathological cell metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the diseases and pathological cells (Hassan, Waseem, et al. 2017; Chaudhary, Priya, et al. 2023; Mishra, Yachana, et al. 2022). Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species.

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels (Singh, S., et al. 2019). The global prevalence of diabetes is estimated to increase from 4% in 1995 to 5.4% by the year 2025 (King, H., et al. 1998). Screening of various bioactive compounds from plants has led to the discovery of new medicinal drug which have efficient protection and treatment roles against various diseases of memory, leucoderma, jaundice, tumours, mouth troubles, sore-eye, fever, and gonorrhoea (Mehra, S., et al. 2023); Kumari, N., et al. 2021). *Adhatoda vasica* is useful in treating bronchitis, tuberculosis and other lung and bronchiole disorders with cough and other symptoms of colds (Gangwar, A. K., & Ghosh, A. K. (2014)).

The plant has pungent and astringent taste. It is cold in action. It normalizes kapha and pitta and improves the voice (Clifford, T. (1977)). It is useful in ridding the patient of coughing and asthma and can be given as a cure in any disease with which these symptoms are associated. It is beneficial to the tuberculosis patient (Sharma, D. K., & Jain, V. K. 2022).

The principle constituents of Vasaka are its several alkaloids, the chief one being vasicine (Kharel,

R. 2010). The leaves contain two major alkaloids called vasicine and vasicinone2, 3 (Srivastava, S., et al. 2001). Recent studies on vasicine demonstrated bronchodilatory efficacy both in vitro and in vivo that was comparable to theophylline. The combination of both alkaloids had strong bronchodilatory effects. Additionally, vasicine has potent respiratory stimulant properties. Additionally, thrombopoietic (platelet-increasing) action associated with vasicine has been reported (Sharma, A., et al. 2018; Varma, A., Padh, H., & Shrivastava, N. 2011; Liu, Wei, et al. 2015). Uterine stimulant activity and moderate hypotensive activity of the alkaloids have been observed. The leaves of Vasaka are rich in vitamin C, carotene and an essential oil. A study showed that *Mycobacterium tuberculosis* was inhibited by the essential oil (at a specific concentration). (Kumar, K. et al.2010;). The juice obtained from the leaves can be used to treat bloody stool and mucus in stool. The dry leaves can be rolled and smoked as a cigar to treat wheezing. The flower of *Adhatoda vasica* can be used to treat ailments of eye. For this, the flowers are slightly shown on fire and then placed on the eyelids. Regular application is said to cure eye irritation and other minor ailments. (Khan, I., et al.2017).

## II. METHODOLOGY

- 1) **Preparation of extract:** (Singh, Lav, et al. 2023;)
- 2) **Phytochemical screening:** Phytochemical studies were carried out for methanol extracts of *Adhatoda vasica* leaves to detect the presence of different phytochemical constituents like steroids, terpenoids, tannins, flavonoids, saponins, glycosides, amino acids etc. by using standard procedures (Pawar, K. et. al. 2019)
- 3) **Column chromatography:** The wet slurry method, which involves making a solution of silica gel with water in this case in a beaker and then adding it to the column until it is about three-fourths filled (glass wool was then pushed into the column to settle on top of the packed silica gel), was used to pack the column, which measures 25 cm by 25 cm. Continuously pouring n-butanol, acetone, and water in a 5:2:3 ratio was done into the column, which was then allowed to drain. About 10 fractions, each containing 5–6 ml, were then collected in sterile centrifuge tubes. The fraction eluted on the column was examined by TLC for the presence of active chemicals using the same solvent solution. (Saha, M., & Bandyopadhyay, P. K. 2020)
- 4) **Thin layer chromatography (TLC):** Fraction eluted on column was subjected to TLC as per conventional one dimensional ascending method using silica gel (60F254 MERCK) pre-coated plate, were observed under various wavelength at 254nm and 366nm for band detection (Padma, R. 2009). Colour of the spot and pattern were observed and RF value were calculated using formula:

$$RF \text{ (Retention factor)} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent front}}$$

**5) Anti-diabetic activity: Inhibition of alpha amylase enzyme:**

**Standard maltose curve:** 0.2 – 1ml of standard maltose (1mg/ml) was taken into different tube. Make the volume to 1ml in each case with distilled water. Added 1 ml of DNSA (Dinitro salicylic acid) reagent to each tube and then place all the tubes in boiling water bath for 15 mins. Add 8 ml of D/W in each tube and mix the content then read the absorbance of the solution in calorimeter at 570 nm against blank solution.

**Alpha amylase inhibition assay.** Blank was measured by taking 1ml of phosphate buffer. Control was measured by taking 0.5 ml of phosphate buffer. The solution was taken treated with 0.5 ml of alpha amylase (0.5mg/ml). The solution was incubated at 25° C for 10 mins. Added 0.5 ml of 1% starch solution in 0.02 M Sodium phosphate buffer of pH 6.9 to all tubes and then incubated at 25° c for 10 mins. The reaction was stopped by DNSA and the reaction mixture was kept in boiling water bath for 5 mins cooled to RT. The solution was mixed with 8ml distilled water. Read the absorbance of the solution in calorimeter at 570nm against blank solution (Pillai, R. et al. 2022). Amount of maltose produced is calculated using standard maltose curve and enzyme activity is calculated by using formula.

$$\text{Enzyme activity} = \frac{\text{Amount of maltose formed} \times 2}{10 \times 342}$$

**Reducing power assay:** Reducing power assay was evaluated according to the method of (Bajpai, V. *et.al.*, 2015). Different concentration of partial purified methanol fraction of *Adhatoda vasica* (100-500µg/ml) was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of potassium ferricyanide followed by incubation at 50°C for 20 minutes, then it was cooled rapidly. 1 ml of 10% trichloro acetic acid was added and vortexed. 3. This incubation mixture was centrifuge at 3000 rpm for 10 mins. The supernatant was taken out and mixed with 2 ml of distilled water and 0.5 ml of 1 % ferric chloride. 4. After incubation for 10 minutes, the absorbance was measured at 700nm. Higher absorbance of the reaction mixture indicates the reducing potential of partial purified fraction.

**III. RESULTS AND DISCUSSION**

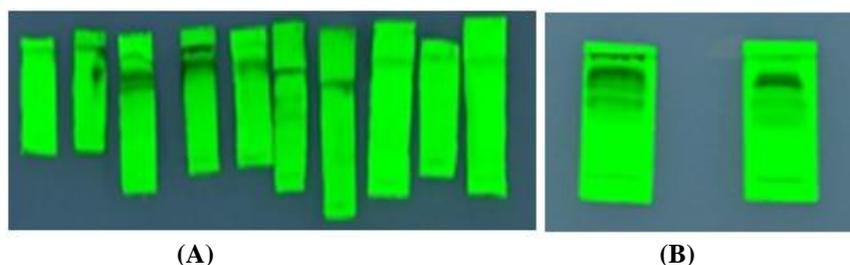
**3.1 Column chromatography and TLC studies:**

Thin layer chromatographic studies of methanol extract of *Adhatoda vasica* was done by using silica gel F254 (MERCK) plate. Solvent system n-butanol: Acetone: water (5:2:3) was used for separation of compound. Fraction eluted on column chromatography showing different band pattern at 254 nm and 366nm. Spot were characterized by Rf value under UV light are demonstrated in Table no. 1 and figure number 1 and 2 respectively.

**Table No. 1: Column chromatography and TLC study of *Adhatoda vasica* methanol fraction with Rf value**

Fraction No.	Solvent System	Number of Spot Detected		Rf Value	
		254 nm	366nm	254nm	366nm
1	Water	-	-	-	-
2	Water	1	1	0.70	-
3	n-butanol: Acetone: water	1	-	0.76	-
4	n-butanol: Acetone: water	1	-	0.86	-
5	n-butanol: Acetone: water	1	-	0.75	0.53
6	n-butanol: Acetone: water	1	2	0.74	0.38
7	n-butanol: Acetone: water	-	2	-	-
8	n-butanol: Acetone: water	-	-	-	-
9	n-butanol: Acetone: water	-	-	-	-
10	n-butanol: Acetone: water	-	-	-	-

**Absence of spots:**



**Figure. No. 1: TLC plates of samples visualized under 254 nm of (A) column eluted fraction & (B) crude methanolic extract of *Adhatoda vasica* in same solvent system.**

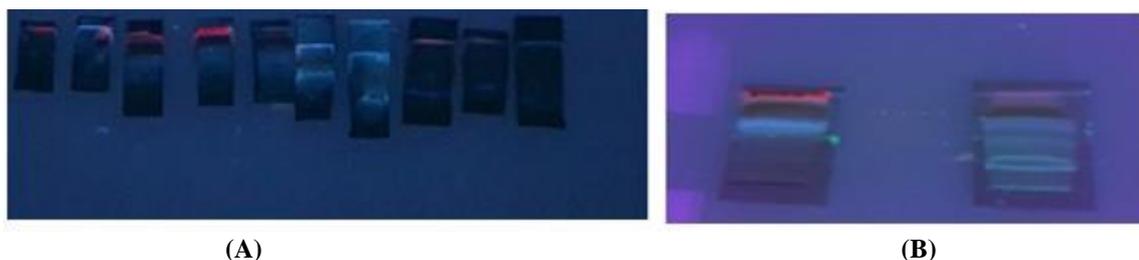


Fig. No. 2: TLC plates of samples visualized under 366 nm of (A) column eluted fraction & (B) crude methanolic extract of *Adhatoda vasica* in same solvent system.

### 3.2 Estimation of protein content of eluted fractions of column chromatography using Nano Drop spectrophotometer.

The protein estimation has been eluted by using column chromatography using Nano Drop spectrophotometer. The result regarding fraction number with protein concentration is shown in table no. 2 and figures no 3. We got highest protein concentration on fraction number 10 and lowest protein concentration at fraction number 4 respectively.

Table No. 2: Determination of Protein concentration by Nanodrop technique

Fraction no	Protein concentration
1 (water eluted)	-0.051
2 (water eluted)	-10.972
3 (solvent eluted)	-15.441
4 (solvent eluted)	-21.124
5 (solvent eluted)	8.476
6 (solvent eluted)	13.593
7 (solvent eluted)	15.453
8 (solvent eluted)	19.275
9 (solvent eluted)	20.440
10 (solvent eluted)	26.289

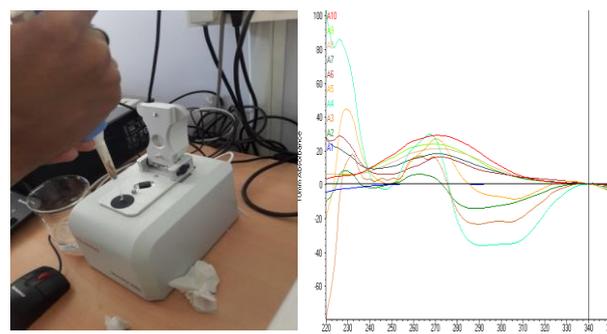


Figure No. 3: Determination of protein concentration of column eluted fractions of column chromatography of *Adhatoda vasica*

### 3.3 Phytochemical Analysis:

Qualitative Phytochemical investigation for extracts of *Adulsa (Adhatoda vasica)* leaves with various specific reagent showed the presence of steroids, flavonoids, coumarous, terpenoids, terpenoids, emodins, alkaloids, phenols and Quinone's Methanolic extract of *Adhatoda vasica* leaves contain maximum number of phytoconstituents. The results of phytochemical screening were showed in the Table No. 3.

Table No. 3: Phytochemical constituents present methanol extracts & partially purified fractions of *Adhatoda vasica*

Sr. No.	Name of phytochemicals	Test	Inference	Observation
1	Alkaloid	Add 2ml of extract to 2N HCL decand aqueous layer formed and few drop of Mayer's reagent	Cream precipitate observe indicating the presence of alkaloid	Cream precipitate was observed
2	Phenolic compounds	Compounds-Add 3-5 drops of 5% FeCl <sub>3</sub> solution to 2ml of extract	Formation of deep blue colour	Deep blue colour was observed
3	Flavonoids	In 2ml of extract, add 2-5 drops of 1N NaOH	Formation of yellow orange colour	Yellowish orange colour seen
4	Saponins	Add 2ml of extract with 6ml of water in a test tube	Observe for persistent foam	Observation of persistent foam
6	Tannins	Add 2ml of aqueous extract with 2ml of distilled water and few drops of FeCl <sub>3</sub>	Formation of green precipitate	Green precipitate was observed
7	Leucoanthocyanins	Add 5ml of aqueous extract to 5ml of Isoamyl alcohol.	Upper layer appears red in colour	Red colour was not observed

8	Quinons	Add 2ml of extract with concentrated HCl	Formation of yellow precipitate	Yellow precipitate was observed
9	Coumarin	Add 3ml of 10% NaOH to 2ml of aqueous extract	Formation of yellow colour	Yellow colour was observed
10	Steroid	Dissolve 1ml of extract in 10ml of chloroform and add equal volume of concentrated H <sub>2</sub> SO <sub>4</sub>	The upper layer turns red and H <sub>2</sub> SO <sub>4</sub> layer shows yellow green fluorescence	The upper layer turns red and H <sub>2</sub> SO <sub>4</sub> layer yellow green fluorescence
11	Emodins	Add 2ml of extract with concentrated HCl	Formation of yellow precipitate	Yellow precipitate was observed
12	Phlobatanin	Add 2ml of aqueous extract to 2ml of 1% HCl and boil the mixture.	Deposition of red precipitate	Red precipitate was not observed
13	Anthocyanin	Add 2ml of aqueous extract to 2ml of 2N HCl and Ammonia	Appearance of pink-red urns Blue- violet	Pink-red colour turns Blue-voilet

3.4 *In vitro anti-diabetic activity:*

3.4.1 *Alpha amylase inhibition assay*

The intestinal digestive enzyme alpha-amylase plays vital role in the carbohydrate digestion. Antidiabetic therapeutic approach reduces the post prandial glucose level in blood by the inhibition of

alpha- amylase enzyme. The *In vitro* alpha amylase inhibitory studies demonstrated that *Adulsa (Adhatoda vasica)* has well antidiabetic activity. The % inhibition at conc.100-500µg/ml of fraction show conc. Dependent % inhibition.

Table No. 7: *In vitro* alpha amylase inhibition assay of column eluted fractions of *Adhatoda vasica*.

Concentration µg/ml	Fraction 4		Fraction 5		Standard	
	Absolute	% Inhibition	Absolute	% Inhibition	Absolute	% Inhibition
100	0.115	40.80±0.004	0.107	25±0.005	0.027	43 ±0.05
200	0.251	50±0.004	0.141	43.3±0.002	0.032	59 ±0.05
300	0.281	53.3±0.004	0.204	57.73±0.004	0.053	74.42±0.06
400	0.288	70.71±0.005	0.206	66.14±0.004	0.057	82.23±0.06
500	0.366	79.05±0.003	0.275	77.05±0.003	0.075	85.75±0.08

Values are expressed as mean±SD; Experimental group were compared with control p<0.01, considered extremely significant.

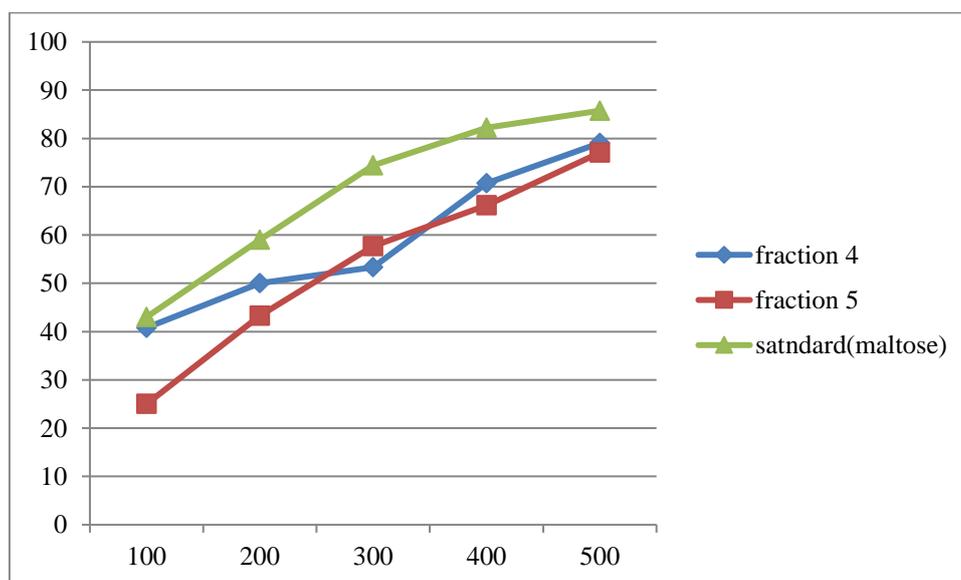


Figure No. 8.: *In vitro* alpha amylase inhibition assay of column eluted fractions of *Adhatoda vasica* . Graph comparing alpha amylase inhibition in column eluted fractions and standard maltose.

3.4.2 Antioxidant activity:

**Reducing power assay:** Reducing power assay was used for determining antioxidant activity in *Adhatoda*

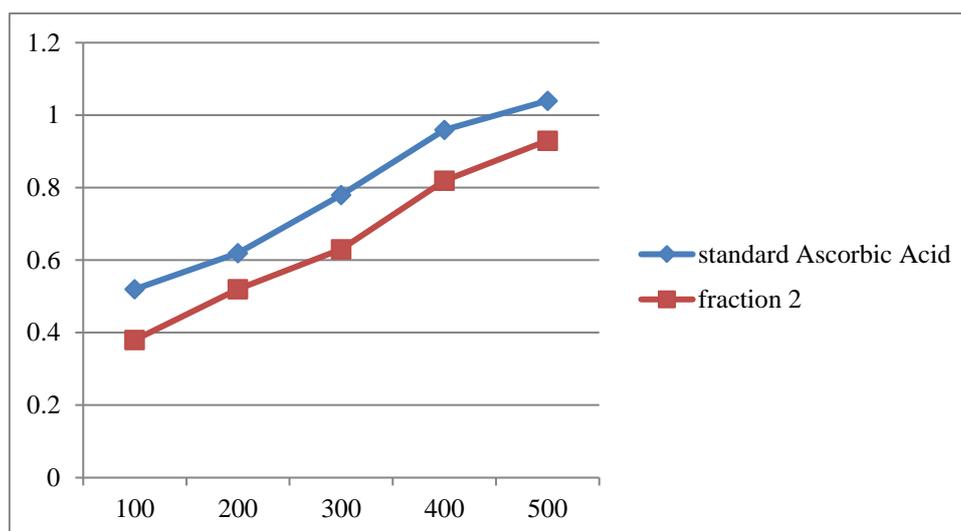
*vasica*. Ascorbic Acid was used as standard. Absorbance was taken 700 nm on spectrophotometer.

**Table No. 8: Reducing power assay to determining antioxidant activity in column eluted fractions of *Adhatoda vasica***

Concentration µg / ml	Standard Abs at 700nm	Test sample abs at 700nm
100	0.52	0.38
200	0.62	0.52
300	0.78	0.63
400	0.96	0.82
500	1.04	0.93

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron. The reducing power ability of column eluted fraction of *Adhatoda vasica* increased constantly with increase in the volume of column eluted fraction from 100-500 µg/ml. fraction no. 2 has higher absorbance 1.04 at 500µg/ml followed by reducing power of

column eluted fraction was compared with ascorbic acid as standard showing higher absorbance 0.93 at 500µg/ml. Absorbance listed in table number 8. Graphical representation for comparison of inhibition between standard ascorbic acid and the column eluted samples of *Adhatoda vasica*.



**Figure No. 9: Reducing power assay of column eluted fraction of *Adhatoda vasica* where absorbance was taken at 700 nm on spectrophotometer.**

**IV. CONCLUSION**

Phytochemical screening reflects the presence of alkaloid, flavonoids, coumarins, terpenoids, steroids, emodins, Quinone's. Column chromatography method was used for purification of bioactive compounds. Thin layer chromatographic study was carried out by using various solvent system of different type of polarity of *n*- butanol, acetic acid and acetone. TLC profiling shows pure band at 254nm and 366 nm. The strong "proteolytic activity" also pointed out in purified fraction of eluted fraction. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation fraction 3, showing highest activity 75% followed by fraction 5 (62.73%), *In vitro* antidiabetic

activity was performed using Alfa amylase inhibition assay. Highest activity were showed in fraction 4 (79.05 %) and fraction 5 (77.05 %) at concentration. 500 µg/ml.

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