ISOLATION, CHARACTERIZATION & HPTLC CHROMATOGRAPHIC FINGERPRINT ANALYSIS OF ACTIVE PHYTOCONSTITUENTS FROM DICHLOROMETHANE EXTRACT OF *DOLICHANDRONE FALCATE* SEEM.

Vaibhav Verma[1*], Rahul Trivedi[2]

ABSTRACT

AIM- The aim of the present investigation is to isolate, characterize and develop chromatographic analysis of dichloromethane extract of *Dolichandrone falcate* Seem. leaves.

MATERIAL & METHODS- The correctly identified plant leaves is dried in shade at room temperature & after 4-5 days, it is formed in powder by mixer grinder. Powdered drug 100gm was weighed and packed in soxhlet. Defatted drug was subjected to extraction with dichloromethane, ethyl acetate, ethanol and finally by using aqueous system. TLC for the separation of various bioactive compounds from bioactive extract, dichloromethane was developed to find out the probable number of compounds present in them. Solvent systems; n-hexane: chloroform: ethanol (5:3:1) were found to be most satisfactory. For the HPTLC analysis, The sample was dissolved in 10 ml of dichloromethane, sonicated for 10 minutes, filtered and applied on TLC plates (5×10 cm) in 2 tracks (4µl for dichloromethane extract) in the form of band. One side of twin trough chamber was charged with the solvent system n-hexane: chloroform: ethanol (5:3:1) and allowed to equilibrate for 10 minutes. Plate was then scanned at 366 nm in case of dichloromethane extracts. In column chromatography, mixture as prepared above was fed very slowly into the column without disturbing the silica bed. Thereafter appropriate solvent system i.e. n-hexane: chloroform: ethanol (5:3:1) was poured into the column for elucidation of components. Elute was collected at the rate of 20 drops per minute and each fraction was about 25 ml.

RESULTS- The spotted plate was then dipped in mobile phase and solvent front was allowed to travel about 70-80% distance on the plate vertically. The compound I and II is white crystalline needle like substance whereas compound III is off-white. The melting point of Compound I, Compound II and Compound III was 133°C, 176°C and 160°C respectively. Mass spectrum of Compound I, II and III showed a parent molecular ion $[M+H]^+$ peak at m/z 414, 412 and 426 respectively which corresponds to the molecular formula $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{38}H_{64}O_2$.

CONCLUSION-Total three compounds were isolated in significant amount and their characterization was done using different spectral techniques based on the following deliberations, to reveal their identity as steroids and triterpenes.

KEYWORDS: Isolation, Characterization, HPTLC Chromatographic Analysis, Dichloromethane Extract, *Dolichandrone falcate* Seem. Leaves

[1*]Research Scholar, Faculty of Pharmacy, Mandsaur University, Mandsaur (MP), 458001
[2]Faculty of Pharmacy, Mandsaur University, Mandsaur (MP), 458001

[*]**Corresponding Author:** Vaibhav Verma
Research Scholar, Faculty of Pharmacy, Mandsaur University, Mandsaur (MP), 458001

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INTRODUCTION

Predictably, insulin dependent diabetes is treated with exogenous insulin and noninsulin dependent diabetes with synthetic oral hypoglycemic agents like Sulphonylurea and Biguanides. However, hormone fails as a curative agent for complications of diabetes and the major drawbacks of insulin therapy are the side effects like insulin allergy, lipodystrophy and lipoatropy, insulin antibodies, altered metabolic control, autoimmunity and other late complications like morphological changes in kidneys and severe vascular complications (Pepato *et al*., 2005). Similarly, oral hypoglycemic drugs have many side effects such as nausea, vomiting, cholestatic jaundice, aplastic and hemolytic anemia's, generalized allergic reactions, dermatological reaction etc (Mallick *et al*., 2007).

Traditionally, there are various herbs are being used for the treatment of diabetes mellitus, from which merely some have been evaluated as per the modern system of medicine. From these plants only plant extracts have been prepared and evaluated for its Antihyperglycaemic activity. Most of the reported plants seem to act directly on pancreas and stimulate insulin release in the blood (Al-Qattan *et al*., 2008). Some will favorably alter the activities of regulatory enzymes of glycolysis, gluconeogenesis and other pathways by acting directly on tissues like liver, muscle and fat.Chemical constituents of these plants are known to possess wide range of medicinal properties (Shabeer *et al*., 2009).

As per earlier reports, very low fat and lower glycemic index diet, regular exercise and weight control has evident potential to control type II diabetes but major proportion of population suffering from diabetes continue to eat whatever they want, avoiding exercise and stay fatty. Treatment of insulin dependent diabetes mellitus with insulin and non insulin dependent diabetes mellitus with synthetic oral hypoglycemic agents such as Sulphonylurea, Biguanides, thiazolidinediones etc. is currently available. However, many of these oral antidiabetic agents have a number of serious adverse health effects. Therefore, the search for more effective and safer hypoglycemic with greatest effect on postprandial hyperglycemia including amylin analogues, insulin and α-Glucosidase has continued to be an important area for investigation (Sutherland and Hoehns, 2004). In our previous study, dichloromethane extract of leaves of *Dolichandrone falcate* Seem. showed the prominent *In-vitro* anti-diabetic activity in two different enzymatic assay methods. Here, an attempt was made to identify the possible responsible active phytoconstituents for the anti-diabetic activity.

MATERIAL & METHODS

Collection and authentication of the plant leaves

The leaves of *Dolichandrone falcate* Seem. was collected from outfield near herbal garden of College of Pharmacy, India during the month of July that shows the green color with rough surface. Plant was identified by the Research Officer, Botany and herbarium specimen was submitted in Department of Pharmacognosy for the future reference.

Successive extraction methods

The correctly identified plant leaves is dried in shade at room temperature & after 4-5 days, it is formed in powder by mixer grinder. Powdered drug 100gm was weighed and packed in soxhlet. The drug was continuously extracted with petroleum ether for about 72 hours. Complete defatting was measured by placing a drop form the thimble on a filter paper give any oily spot. The mark was dried in air to remove traces of petroleum ether. Defatted drug was subjected to extraction with dichloromethane, ethyl acetate, ethanol and finally by using aqueous system. The % Yield of the petroleum ether, dichloromethane, ethyl acetate, ethanol & aqueous extract of *Dolichandrone falcate* Seem. was calculated **(**Sarija *et al*., 2014**)**.

Chromatographic study of dichloromethane extract

Thin Layer Chromatography of dichloromethane extract

TLC for the separation of various bioactive compounds from bioactive extract, dichloromethane was developed to find out the probable number of compounds present in them. On the pre coated TLC plate, test samples were applied in the form of spots with the help of fine capillary. Spots were marked on the top of the plate for their identification. Rectangular glass chambers were used for chromatography. To avoid insufficient chamber saturation and undesirable edge effect, a smooth sheet of filter paper was placed in TLC chamber and was allowed to be in the developing solvent. A number of developing solvent systems were tried during the study. Each time plate was sprayed with Anisaldehyde sulphuric acid and vanillin sulphuric acid and heated at 115^0C for 5 minutes. The solvent system in which there is a satisfactory resolution was taken as a final solvent system. Solvent systems; n-hexane: chloroform: ethanol (5:3:1) were found to be most satisfactory. After development of plates,

they were air-dried and number of spots, color and R_f values were recorded (Wagner *et al*., 1986; Mukherjee, 2002).

R_f value=Distance travelled by solute/Distance travelled by solvent

HPTLC of dichloromethane Extract

Plate was kept in an ascending mode and activated at 120^0C for 1 hour prior to application of sample bands. The sample was dissolved in 10 ml of dichloromethane, sonicated for 10 minutes, filtered and applied on TLC plates (5×10 cm) in 2 tracks (4µl for dichloromethane extract) in the form of band. One side of twin trough chamber was charged with the solvent system n-hexane: chloroform: ethanol (5:3:1) and allowed to equilibrate for 10 minutes. The spotted plate was then dipped in mobile phase and solvent front was allowed to travel about 70-80% distance on the plate vertically. Plate was then removed from chamber and dried.

Plate was then scanned at 366 nm in case of dichloromethane extracts. Photos of the plate were taken at 366nm. Number of spots, color, R_f values and % relative areas were recorded (Wagner *et al*., 1986; Mukherjee, 2002).

Bioactivity guided isolation from dichloromethane extract

Wet packing method was adopted for packing the column. 10 gm of extract was mixed with 50 gm of silica gel and a very small amount of an appropriate solvent. This mixture was triturated in a mortar till a homogenous and dry free flowing mixture was obtained. The mixture as prepared above was fed very slowly into the column without disturbing the silica bed. Thereafter appropriate solvent system i.e. n-hexane: chloroform: ethanol (5:3:1) was poured into the column for elucidation of components. Elute was collected at the rate of 20 drops per minute and each fraction was about 25 ml. Each fraction was subjected to preparative TLC. The fraction with same R_f were pooled together and concentrated to obtain pure compounds or a mixture of 2-3 compounds. Elutes from column chromatography were rechromatogramed or subjected to preparative TLC as required to obtain pure compounds (Shukla *et al*., 2012).

Evaluation of Isolated Compound

Melting point determination

Melting point was determined using melting-point apparatus by capillary tube methods. A few crystals of the compound were placed in a thin walled capillary tube 10-15 cm long, about 1 mm in inside diameter, and closed at one end. Then the capillary, which contains the sample, and a thermometer are then suspended so they can be heated slowly and evenly. The temperature range over which the sample is observed to melt is taken as the melting point.

Test for Phytochemical

For the evaluation of steroids, a few crystals of isolated compound (i.e. compound I, II and III) were dissolved in chloroform and 1-2 ml acetic anhydride was added with 2 drops of conc. H2SO4 from the side of the tube. Red colour, then blue and finally green color appears.

Spectral analysis and Structure elucidation

Tools most widely used for structure elucidation of natural products are Fourier transform infrared spectroscopy (FTIR), mass spectroscopy (MS) and nuclear magnetic resonance (NMR). With these tools the structures of most natural products can be determined. FTIR identifies chemical bonds in a molecule fingerprint that can be used to easily screen and scan samples for many different components. It detects functional groups and characterizes covalent bonding information. NMR has become increasingly more important in structure elucidation. It has almost completely replaced degradation studies in the determination of novel structures, and in many cases synthesis is no longer necessary as a structural proof. The ^{1}H NMR produces spectrum produced by signals that indicate the number of different types of protons and the integration specifies the ratios of number of protons. The integration of these molecular spectroscopic techniques provides complementary data regarding a molecule's structure, and when used together they prove very effective in identification of unknown compounds.

Infrared (IR) spectra were recorded on a Shimadzu (Japan) 8400 S FT-IR spectrophotometer model using potassium bromide pellets (υ_{max} in cm^{-1}). ^{1}HNMR spectra were recorded on Brucker multinuclear FT NMR spectrometer model AV-400, 400 MHz using deuterated-chloroform or deuterated dimethylsulfoxide-containing tetramethylsilane (Me_4Si) as internal standard (chemical shifts in δ, ppm).

RESULTS

Thin Layer Chromatography

A number of developing solvent systems were tried during the study. Each time plate was sprayed with Anisaldehyde sulphuric acid and vanillin sulphuric acid and heated at 115^0C for 5 minutes. The solvent system in which there is a satisfactory resolution

was taken as a final solvent system. Solvent systems; n-hexane: chloroform: ethanol (5:3:1) were found to be most satisfactory. After development of plates, they were air-dried and number of spots, color and R_f values were recorded.

Table No. 1: TLC summary of dichloromethane extract

S No.	Extract	Solvent systems	Detecting reagents	Color	No. of spots	R_f value of Spots
1	Dichloromethane	n-hexane: chloroform: ethanol (5:3:1)	Anisaldehyde sulphric acid, heated at 100^0C for 5 Min.	Green & yellow	4	0.34, 0.41. 0.52, 0.64, 0.67

HPTLC of Dichloromethane Extract-

TLC plate was kept in an ascending mode and activated at 120^0C for 1 hour prior to application of sample bands. The sample was dissolved in 10 ml of chloroform, sonicated for 10 minutes, filtered and applied on TLC plates (5×10 cm) in 1 tracks (4µl for dichloromethane extract) in the form of band. One side of twin trough chamber was charged with the solvent system n-hexane: chloromethane: ethanol (5:3:1) and allowed to equilibrate for 10 minutes. The spotted plate was then dipped in mobile phase and solvent front was allowed to travel about 70-80% distance on the plate vertically. Plate was then removed from chamber and dried. Plate was then scanned at 366 nm in case of dichloromethane extract. Photos of the plate were taken at 366nm.

Table No 2: R_f values & relative percentage of compounds from dichloromethane extract

S No.	Volume	Peak	Start R_f values	Start Height	Max Height	Max %	End R_f	Area	% Area
1	4 µL	1	0.04	90.6	90.6	8.02	-0.02	452.3	0.68
2	4 µL	2	0.02	0.3	11.1	0.98	0.01	76.8	0.12
3	4 µL	3	0.07	31.8	171.9	15.21	0.18	8086.0	12.20
4	4 µL	4	0.18	164.2	174.7	15.45	0.20	2699.9	4.07
5	4 µL	5	0.24	172.0	290.7	25.71	0.51	36334.6	54.84
6	4 µL	6	0.52	67.3	171.6	15.18	0.63	8845.0	13.35
7	4 µL	7	0.63	85.7	102.2	9.05	0.73	4747.3	7.17
8	4 µL	8	0.77	6.3	22.4	1.98	0.80	403.3	0.61
9	4 µL	9	0.85	21.8	95.3	8.43	0.98	4611.4	6.96

Characterization and Identification of compounds

All the three compounds gave positive tests for steroids and alcohols given by Compound I, II and III. The compound I and II is white crystalline needle like substance whereas compound III is off-white. The melting point of Compound I, Compound II and Compound III was 133°C, 176°C and 160°C respectively. Mass spectrum of Compound I, II and III showed a parent molecular ion $[M+H]^+$ peak at m/z 414, 412 and 426 respectively which corresponds to the molecular formula $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{38}H_{64}O_2$. The IR, ^{1}H-NMR and ^{13}C-NMR data of Compound I, II and III are as follow:

Compound I

IR (KBr) 3460, 2868, 1731, 1644, 1512, 1455, 1421, 1350, 1298, 1249, 1201, 1108, 950, 849, 726, 552cm$^{-1.}$ ^{1}H NMR (400 MHz, Chloroform) δ 5.48 (s, 5H), 3.42 (s, 5H), 2.25 (s, 4H), 2.02 (s, 4H), 1.88 (d, *J* = 18.0 Hz, 9H), 1.77 (s, 4H), 1.70 (d, *J* = 9.2 Hz, 11H), 1.66 – 1.56 (m, 15H), 1.48 (dd, *J* = 15.0, 7.2 Hz, 21H), 1.42 – 1.12 (m, 72H), 1.16 – 1.12 (m, 18H), 1.15 – 1.08 (m, 27H), 1.08 – 0.90 (m, 92H), 0.85 (s, 6H), 0.73 (s, 4H). ^{13}C NMR (100 MHz, Common NMR Solvents) δ 141.20 (s), 121.64 (s), 71.05 (s), 57.01 (s), 56.46 (s), 51.13 (s), 45.02 (s), 43.08 (d, *J* = 9.0 Hz), 39.72 (s), 37.77 (s), 37.16 (s), 36.72 (s), 35.41 (s), 32.66 (s), 32.31 (s), 31.60 (s), 31.13 (s), 28.32 (d, *J* = 4.1 Hz), 24.97 (d, *J* = 2.6 Hz), 21.27 (s), 19.99 – 19.67 (m), 18.84 (d, *J* = 13.2 Hz), 13.00 (s), 12.11 (s).

Compound II

IR (KBr) 3462, 3001, 2888, 1957, 1731, 1643, 1531, 1425, 1421, 1350, 1298, 1220, 1112, 950, 841, 715, 552cm^{-1}. ^{1}H NMR (400 MHz, Chloroform) δ 5.48 (s, 7H), 5.26-5.19 (m, 13H), 3.42 (s, 6H), 2.25 (s, 5H), 2.08 (s, 6H), 2.02 (s, 5H),

1.88 (t, J = 9.7 Hz, 19H), 1.80- 1.68 (m, 19H), 1.68-1.53 (m, 28H), 1.51-1.44 (m, 19H), 1.34 (dd, J = 10.4, 8.1 Hz, 25H), 1.26 (d, J = 2.1 Hz, 3H), 1.25-1.10 (m, 51H), 1.02-0.93 (m, 95H), 0.85 (s, 5H), 0.73 (s, 5H).^{13}C NMR (100 MHz, Common NMR Solvents) δ 141.20 (s), 137.38 (s), 130.38 (s), 121.64 (s), 71.05(s), 57.01 (s), 55.58 (s), 51.90 (s), 51.13 (s), 43.61 (s), 43.03 (s), 40.65 (s), 39.72 (s), 37.77 (s), 37.16 (s), 32.66 (s), 33.31 (s), 31.80 (s), 31.60 (s), 29.20 (s), 24.99 (s), 23.15 (s), 21.77 (s), 20.10- 19.33 (m), 18.91 (s), 13.00 (s), 11.58 (s).

Compound III

IR (KBr) 3442, 3056, 2860, 2693, 2237, 2159, 1963, 1731, 1643, 1512, 1483, 1419, 1359, 1344, 1279, 1220, 1147, 1049, 946, 842, 657, 537cm^{-1}. 1H NMR (400 MHz, Chloroform) δ 5.40 (s, 7H), 3.47 (s, 7H), 2.80 (s, 6H), 2.23 (s, 7H), 2.02 (s, 5H), 1.96 (dd, J = 9.5, 1.7 Hz, 27H), 1.85 (d, J = 19.4 Hz, 13H), 1.77 (s, 5H), 1.68 (dd, J = 24.5, 5.8 Hz, 1H), 1.74 – 1.21 (m, 122H), 1.21 – 1.16 (m, 18H), 1.03 – 0.99 (m, 41H). ^{13}C NMR (100 MHz, Common NMR Solvents) δ 143.02 (s), 125.43 (s), 78.57 (s), 57.72 (s), 54.69(s), 46.52 (s), 42.14 (s), 40.20 (s), 39.67 (s), 38.71 (s), 38.39 (s), 38.04 (d, J = 8.4 Hz), 37.47 (s), 34.06 (s), 32.86 (s), 32.39 (s), 29.16 (s), 27.68 (d, J = 8.7 Hz), 25.88 (s), 24.13 (s), 23.89- 23.67 (m), 23.36 (s), 19.61 (s), 18.79 (s), 18.02 (d, J = 16.2 Hz), 17.05 (s).

As all the three compounds showed positive results for steroids and alcohols, they were assumed to be a compound containing steroidal nucleus. The molecular formula and melting point of compound I, II and III were in good agreement with the molecular weight and melting point given for β-sitosterol, stigmasterol and α-amyrin in the literature.

In compound I, IR absorptions bands was appeared at 3460 cm^{-1} (OH), 2868 cm^{-1} (CH2), 2867.38 cm-1 (CH), 1644 cm^{-1} (C=C), 1108 cm^{-1} (C-O). The ^{1}H- NMR spectrum shows that H-2 proton appeared at δ 3.42 as a singlet and H-7 olefinic proton shows a singlet at δ 5.48. Also, six methyl protons appeared as multiplet at δ 1.18, δ1.126, δ 1.031, δ 1.029, δ 1.029 and δ 0.99. The ^{13}C-NMR has shown recognizable signals at 141.20 and 121.63ppm, which corresponds to double bond at C-4 and C-7 double bonds respectively. The δ value at 71.04 ppm is due to C-2 β- hydroxyl group. The signal at δ 18.90 and δ 12.99 ppm corresponds to angular carbon atom at C-26 and C-28 respectively. This assignment of ^{13}C-NMR is in good agreement for the structure of β-sitosterol. Overall, the above spectral data's as well as available literatures supported that the isolated compound is β-sitosterol.

In Compound II, IR absorptions bands was appeared at 3462 cm^{-1} (OH), 3218 cm^{-1} (cyclic olefinic – HC= CH- stretching), 3001 cm^{-1} (=CH stretching) and 2888 cm^{-1} assigned to C-H str. The ^{1}H- NMR spectrum shows that H-2 proton appeared at δ 3.42 as a singlet and H-7 olefinic proton shows a singlet at δ 5.48. H-20 and H-21 two olefinic protons appeared downfield as multiplet at δ 5.26 and 5.19 respectively. Also, six methyl protons appeared as multiplet at δ 1.18, δ1.126, δ 1.031, δ 1.029, δ 1.029 and δ 0.99 which were quite similar with β-sitosterol as mentioned by Habib et al. in 2007. The ^{13}C-NMR has shown recognizable signals at 141.20 and 121.63ppm, which corresponds to double bond at C-4 and C-7 double bonds respectively as well as it also represent signals at 137.37 and 130.38 ppm, which shows one more double bond in between C-20 and C-21. The δ value at 71.04 ppm is due to C-2 β-hydroxyl group. The signal at δ 18.90 and δ 12.99 ppm corresponds to angular carbon atom at C-26 and C-28 respectively.

In Compound III, IR absorption bands were appeared at 3442 cm^{-1} indicating the presence of hydroxyl group, 3056 cm^{-1} (C-H str. in CH_3), 2860 cm^{-1} (C-H str. in CH_2), 1731 cm^{-1} (C=O str.), 1483 cm^{-1} (C-H def. in CH_3), 1359 cm^{-1} (C-H deformation in gem dimethyl), 842 cm^{-1} (=C-H out plane bending). The ^{1}H- NMR spectrum shows that H-2 proton appeared at δ 3.47 as a multiplet and H-13 olefinic proton shows a singlet at δ 5.40. Also, eight methyl protons appeared as singlet as well as multiplet at δ1.13, δ 1.02, δ 1.01, δ 0.96, δ 0.94, 0.88 and δ 0.88. The ^{13}C-NMR has shown recognizable signals at 143.02 and 125.43ppm, which corresponds to double bond at C-12 and C-13. The δ value at 78.56 ppm is due to C-2 β-hydroxyl group. The peaks also showed that the isolated compound had eight methyl group, ten -CH_2 group and four –CH groups. The results were compared with the available literature and confirmed the presence of α-amyrin (Srivastava *et al*., 2012).

DISCUSSION

Bioactive extract was then tested chemically to know the presence of different chemical constituents. TLC and HPTLC studies were also performed to know the number of constituents present in extract and to establish finger print profile. In the present investigation, phytochemical screening showed the presence of steroids, terpenoids in dichloromethane extract. TLC findings were in agreement with the data of qualitative chemical tests and the spots

characteristic for steroids, terpenoids were observed (Chaudhari *et al*., 2012).

Bioactivity guided isolation was performed to separate Phytoconstituents from bioactive dichloromethane extract by applying variety of mobile phases. The dichloromethane extract was subjected to column chromatography using silica gel (60-120 mesh size), and elution done sequentially with Petroleum ether: dichloromethane (DCM), (10% increments of DCM) and finally with DCM. After that extract was eluted with chloroform: ethanol gradient beginning with 100:10 and further eluted with increment of 5% of ethanol, and was finally with 100% ethanol. The elutes were collected in fractions of 25 ml each and were continuously monitored by TLC (n-Hexane: chloroform: Ethanol, 5:3:1) for homogeneity and the similar fractions were pooled together. One hundred and sixty two fractions (each 25 ml) were collected. Fraction 1-25, eluted with hexane and chloroform in the ratio of 9:1 gave several spots in TLC that were inseparable by further processes and was give positive test for fatty material hence discarded. Fraction 26-53, eluted with hexane and chloroform in the ratio of 8:2 were mixed together to give fraction A (Burant *et al*., 1994). Fraction A was further re-chromatographed with a mixture of hexane and ethyl acetate in the ratio of 9:1 to yield compound I. Fraction 55 -83, eluted with hexane and DCM in the ratio of 6:4 were mixed together to give fraction B. Fraction B was rechromatographed with hexane and chloroform in the ratio of 8:2 and on addition of cold diethyl ether gave a precipitate which on successive washing with ether gave compound II. Fraction 104-123, eluted with chloroform and ethanol (9:1) were mixed together to give fraction C, which was crystallized out as compound III on addition of a few drop of cold ethanol. Further fractionization with chloroform and ethanol yields fractions with no spot on TLC (Cho *et al*., 2005).

CONCLUSION

Total three compounds were isolated in significant amount and their characterization was done using different spectral techniques (^{1}H-NMR, Mass, IR and UV analyses) based on the following deliberations, to reveal their identity as steroids and triterpenes. The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of diseases.

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