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COMPARATIVE EVALUATION OF BIOACTIVE COMPOUNDS IN *Hibiscus sabdariffa* AND *Syzygium samarangense* JUICE EXTRACTS

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ABSTRACT

There is growing interest in the chemical composition of plants towards discovery of more effective bio-therapeutic agents. Six bioactive compounds were evaluated from *Hibiscus sabdariffa* and *Syzygium samarangense* juice extracts. Both juices had high amounts of saponins, with *Syzygium samarangense* having higher content (4.77%) than *Hibiscus sabdariffa* (1.46%). Microbial counts in the juices were in the range of 10^4 cfu ml^{-1} of ethanolic extract. The juice extracts showed significant ($P \leq 0.05$) antimicrobial activities against *Escherichia coli, Salmonella typhi* and *Candida albicans*, implying that the juices possess both antibacterial and antifungal properties. The results give substantial scientific credence to the current therapeutic uses of these plants.

Key Words: Antimicrobial, bioactive compound, *Candida albicans, Escherichia coli, Salmonella typhi*

RÉSUMÉ

La composition chimique des plantes s'avère d'une importance capitale dans la découverte des agents bio-thérapeutiques plus efficaces. Six composés bioactifs des jus extraits de *Hibiscus sabdariffa* et *Syzygium samarangense* étaient évalués. Les deux extraits de jus contenaient tous de quantités plus élevées de saponine, avec 4.77 et 1.46 % pour *Syzygium samarangense* et *Hibiscus sabdariffa*, respectivement. Le dénombrement microbiendans les jusavoisinait 104 cfu ml-1 d'extrait d'éthanol. Les extraits de jus ont montré des activités antimicrobiennes significatives ($P \leq 0.05$) contre *Escherichia coli, Salmonella typhi* et *Candida albicans*, signifiant que les deux jus possèdent tous des propriétés antimicrobiennes et antifongiques. Les résultats font montre d'une crédibilité scientifique substantielle eu égard aux usages thérapeutiques courants de ces plantes.

Mots Clés: Antimicrobien, composébioactif, *Candida albicans, Escherichia coli, Salmonella typhi*

INTRODUCTION

Plants whose extracts can be used directly or indirectly for the treatment of different ailments are termed medicinal plants. These plants are considered to be a chemical factory for bio-pharmaceuticals as they contain multitudes of naturally occurring chemical compounds (Amrit, 2006). These chemical compounds which may be primary, but often are secondary metabolites, are as varied as the plants from which they are obtained.

Plants also contain secondary metabolites, which are organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms but often play an important role in plant defences (Harbone and Baxter, 1993). These substances are also capable of destroying or inhibiting the growth of microorganisms (Houghton, 2002).

Examples include alkaloids, glycosides, terpenoids, phenols, tannins, flavonoids and saponins. There is growing interest in the chemical composition of plants towards discovery of more effective bio-therapeutic agents (Roja and Rao, 2002). The primary benefit of using plant-derived medicines is that they are readily affordable and accessible (Grunwald, 1995).

A significant proportion of branded medicinal plants in the western world originate from the rich biodiversity in developing countries, especially Africa and Asia. The plants are screened, analysed and used in drug preparations and returned as high priced medicines to developing countries. As a result, many people in developing countries cannot afford such drugs. In Nigeria for example, the high cost of basic and essential drugs force over 60% of the rural population to depend on traditional medicine (Ghani *et al.*, 1989). Such traditional medicines originate from the wide variety of untapped biodiversity in the country.

Previous studies have documented the antimicrobial activities of a number of recognised local medicinal plants (Adebanjo *et al.*, 1983; Akpulu *et al.*, 1994). However, many plants with potential antimicrobial and medicinal properties remain to be investigated. Two of such plants are *Hibiscus sabdariffa* and *Syzygium samarangense*.

All above ground-parts of the Roselle plant (*Hibiscus sabdariffa*) are valued in native medicine (David and Adam, 1985). Renewed interest in the cultivation of Roselle is centred more on its pharmaceutical rather than food potential. Infusions of the leaves are regarded as diurectic, cholerectic, febrifugal and hypotensive. Pharmacognosists in Senegal recommend Roselle extract for lowering blood pressure (Chopra *et al.*, 1986). In experiments with domestic fowl, Roselle extract decreased the rate of absorption of alcohol and so lessened its effect on the system; the basis of its use as a remedy for after-effects of drunkenness (Morton, 1987a). The calyx extract is used in the treatment of debility, hypertension, dyspepsia and heart ailments. The extracts of the leaves and flowers of Roselle are used internally as tonic tea for digestive and kidney functions (Bown, 1995).

Pink wax apple (*Syzygium samarangense*) is available in Nigeria mostly as a home garden plant; hence little research has been done on the fruit. The plant is underutilised and the fruit is eaten sparingly, despite the belief that it has therapeutic values. It is considered useful in preventing diabetes (personal communication). The flowers are astringent and are believed to play a role in halting diarrhoea. The flowers also show weak antibiotic action against *Staphylococcus aureus*, *Mycobacterium smegmatis* and *Candida albicans* (Morton, 1987b). The leaves and seeds of *Syzygium samaragene* have been shown to have antimicrobial activities against some microorganisms like *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Cryptococcus neoformans* (Chandrasekaran and Vankatesalu, 2004).

There is, however, dearth of information on the bioactive properties of the fruit, which could help in providing valid scientific basis for their use as bio-pharmaceuticals. In developing countries, the search for a new drug is centred upon the investigation and screening of medicinal plants for their bioactive constituents. This work is, therefore, aimed at comparatively evaluating the composition of bioactive compounds present in *Hibiscus sabdariffa* and *Syzygium samarangense*.

MATERIALS AND METHODS

Collection of samples. Sun-dried calyces of *Hibiscus sabdariffa* and fresh fruits of *Syzygium samarangense* were purchased from local markets in south-western Nigeria. The samples were kept in sterile polyethylene bags in ice packs and stored under refrigeration (ca. 4 °C) until analysis. Samples were generally analysed within 48 hr.

Preparation of juice extracts. One hundred grammes of dried calyces of *H. sabdariffa*were ground with sterile mortar and pestle, and then transferred into a 500ml beaker. 300 ml of ethanol (80%) was poured into the 500 ml beaker containing the dry calyces for soaking. The beakerwas sealed with aluminum foil and kept at ambient temperature (28±2°C) for 48 hr. Refrigerated samples were usually allowed to cool

to room/ambient temperature before analysis. The samplewas filtered with a sieve and concentrated using hot water bath according to the method of Kubmarawa *et al. (*2005). Approximately 200 g of fresh pink wax apple fruits were blended with 300 ml of ethanol, sieved using a laboratory sieve (≤ 2 mm)and concentrated in a water bath before being subjected to analysis.

Detection of bioactive compounds in juice extracts

Determination of saponins. Octanol (100 ml) was added to 2 g of each extract, shaken for 5 hr to ensure uniform mixing before filtering through a Whatman No.1 filter paper. Twenty millilitres of 40% saturated solution of $MgCO_3$ was added to neutralise the filtrate obtained. The mixture was again filtered to obtain a clear colourless solution. To 1 ml of the clear solution, was added 2 ml of 5% $FeCl_3$ solution before making up to 50 ml with distilled water. The mixture was allowed to stand for 30 min for the blood red colouration to develop. The absorbance of the samples were read with a spectrophotometer (Spectronic 20) at a wavelength of 30 nm and compared with 0-10 ppm standard saponin solutions. Percent saponin present in the samples was calculated as follows:

% Saponin = Absorbance of Sample x Average gradient x Dilution factor/10,000

Determination of alkaloids. Two milliliters of each extract was warmed with 2% H_2S0_4 for 2 min. Each extract was filtered through a Whatman No.1 filter paper and 2 drops of Dragendorff's reagent were added. Orange red precipitation indicated the presence of alkaloids, after which quantification was estimated by mixing 10 g of each sample with 20 ml of alcohol (80%). After thorough mixing, more alcohol was added to make the mixture up to 100 ml, before 1 g magnesium oxide was added. The mixture was digested in a boiling water bath for 1 hr and 30 min. under a reflux air condenser with occasional shaking. The residue was returned to the flask and re-digested for 30 min with 50 ml alcohol, after which the alcohol was evaporated and hot water was added. 2-3 drops of 10% HCl was added and the solution was transferred into a 150 ml volumetric flask and mixed thoroughly with 5 ml each of zinc acetate and potassium ferric cyanide solutions. The mixture was allowed to stand for a 5 min before being filtered through a dry No. 1 Whatman filter paper. Ten millilitres of filtrate was transferred into a separator funnel and the alkaloids present were extracted by shaking vigorously with five successive 30 ml portions of chloroform. The residue obtained was dissolved in hot water and and eventually used for determination of N by the Kjeldahl technique (Lang, 1958). The percentage Nitrogen obtained was multiplied by the conversion factor 6.26 to get percentage total alkaloid (AOAC, 1990).

Determination of tannins. Two milliliters of each extract was mixed with distilled water in a separate tube and heated in a water bath at 40 ^oC.The mixture was filtered through aWhatman No.1 filter paperand20ml of ferric chloride reagent was added to each filtrate to develop a green colour. According to Hagerman (1978)a dark green coloration indicated the presence of tannins.

A total of 100 ml of 4:1 solvent mixture (i.e. 80 ml of acetone + 20 ml of glacial acetic acid)was used to extract tannin from 2 g of each sample. After allowing the mixture to soak for 5 hr, the sample was filtered using a double layered Whatman No.1 filter paper. The filtrate was made up to the 100 ml mark with distilled water and mixed thoroughly. One millilitre of the sample extract was pipetted into 50 ml volumetric flask, then 20 ml distilled water was added. Furthermore, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 were added before mixing thoroughly. The mixture was made up to the mark with water, mixed again and allowed to stand for 20 min. to develop the desired bluish-green colouration, which indicates a positive result for tannins. A standard tannic acid solution of range 0-10 ppm was treated similarly as 1 ml of sample above. The absorbance of the tannic acid standard solution and of the samples was read off a spectrophotometer (Spectronic 20) at a wavelength of 760 nm. The percentage of tannin was calculated using the formula:

$$\% \text{ Tannin} = \frac{\text{Absorbance of Sample x Average Gradient x Dilution factor}}{10{,}000}$$

..... (Equation 1)

Determination of total polyphenols. Ten grammes of samples were soaked in 20 ml of distilled water for 4 days. The samples were filtered using a Whatman No.1filter paper and each filtrate was made up to 100 ml with distilled water. One millilitre of the filtrate from each sample was measured into a test-tube, 3 ml of each 0.008 N potassium hexacyanoferrate (III) and 0.01 N of iron (III) chloride were added into each filtrate. The absorbance of each filtrate was read on a spectrophotometer after 10 min. The percentage total polyphenol was determined using the formula:

$$\% \text{ Total polyphenol} = \frac{\text{Absorbance x Average gradient x Dilution factor x100}}{\text{Weight of sample}}$$

..... Equation 2

Determination of flavonoids. This was done by taking 2 ml of each extract and adding 2 ml of 0.1 N sodium hydroxide concentrationand0.1 N hydrochloric acid. According to this procedure, yellow solutions that turned colorless indicated the presence of flavonoids, which were quantified by extracting 5 g of each sample with 100 ml solvent - 1% aluminum chloride solution in methanol (95%) (Earnsworth*et al.*, 1974). Then the absorbance of the filtrates were read on a spectrophotometer at 380 mm wavelength and compared with 0.10 ppm, 0.15 ppm, 0.20 ppm, 0.25 ppm and 0.30 ppm standards. The slope was calculated from the standard curve as follows:

$$\% \text{ Flavonoid} = \frac{\text{Metre reading x slope x dilution factor x 100}}{\text{Weight of sample}}$$

..... Equation 3

Determination of glycosides. Five grammes of each sample were taken into 250 ml conical flasks along with 50 ml of ethanol (95%) and 20 ml of distilled water. The mixture was sonicated for 5 min before addition of 8 ml of concentrated hydrochloric acid. It was refluxed for 3 hr and cooled to room temperature. Then the extracts were filtered using double layer of filter paper. Each extract was transferred into a two-necked 50 ml flask connected to a steam generator for steam-distillation with saturated sodium bicarbonate solution contained in a 50 ml conical flask for 60 min. One millilitre of starch indicator was added to 20 ml of each distillate and was titrated with 0.2 m of iodine solution. The percentage glycoside was calculated as:

$$\% \text{ Hydrocyanide} = \frac{\text{Titre value x 10 x 0.27}}{\text{1000 x weight of sample}} \times 100$$

…..... Equation 4

Evaluation of microbial flora of juice extracts. Microorganisms associated with the juices were isolated by the pour plate technique using appropriate 10-fold serial dilutions (Lyne and Collins, 1990). Ten millilitres of each sample for microbiological evaluation were aseptically transferred into 90 ml of 0.1% sterile peptone water. They were then shaken thoroughly and appropriate dilutions (up to 10^6) were plated on Plate Count Agar for total viable counts, Nutrient Agar for aerobic mesophiles, MacConkey Agar for enterobacteriaceae, Potato Dextrose Agar for fungi (yeast and molds) and Mannitol Salt Agar for staphylococci. From plates used for enumeration, representative colonies were picked, purified by repeated sub-culturing before being stored on agar slopes at 4 °C for up to 4 weeks or until required for characterisation tests and identification. Moulds were maintained on PDA slants, yeast isolates were kept on yeast extract-dextrose-peptone agar slants while bacteria were kept on nutrient agar slants.

Characterisation and identification of isolated microorganisms. Pure cultures of isolates maintained on appropriate agar slants were characterised by standard procedures (Harrigan and McCance, 1986). Characterisation of isolated bacteria from the juices of *S. samarangense* and *H. sabdariffa* was based on cultural, morphological and biochemical evaluation. For cultural characterisation, colonies randomly picked from inoculated plates were grouped on the bases of their colonial characteristics such as elevation, size, shape, surface, opacity, pigmentation, edge and consistency (Olutiola *et al.*, 1991).

Cultural grouping was followed by microscopic examination of isolates for cellular morphology. Twenty-four-hour broth cultures of bacterialisolates were Gram-stained according to the method of Claus (1992). Light suspensions of cells were made on clean glass slides and spread with a sterile loop over the surface of the slides. Each slide was allowed to air-dry for about 2 min. The slides were then flooded with Hucker's crystal violet reagent for 1 minute. The crystal violet reagent was then washed off the slides by dipping the slides in sterile distilled water for 5 sec. Excess water was rinsed off with stabilised PVP-iodine-KI solution and the slides were flooded with fresh iodine solution for 1 min. The slides were washed again in sterile water for 5 sec as earlier described. The wet slides were then decolorised by immersion for 1 min in n-propanol. They were rinsed with the counter stain and flooded with fresh counter stain for 1 min. The slides were washed again in sterile distilled water for 5 sec as described earlier and allowed to air-dry. The preparations were examined with the oil immersion objective lens of the bright field microscope (Zeiss,USA). According to this procedure, gram-positive cells appeared purple, while gram-negative cells were pink. Cell shape and arrangement were also observed and recorded under this staining technique.

Biochemical analysis for bacterial characterisation included catalase and oxidase activities, nitrate reduction, patterns of sugar utilisation as well as urea and starch hydrolysis (Christensen, 1946; Harrigan and McCance, 1976). Identification of isolates was based on the results obtained from characterisation as analysed with reference to Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986).

For yeast isolates, day-old cultures were stained with cotton blue lacto-phenol and microscopically observed for cell shape, size and sporulation. Yeasts were also observed for their method of vegetative reproduction (budding, fission or filaments). Examination of yeast isolates for pseudomycelium formation on PDA was as described by Barnett *et al.* (1990). Sterilised molten PDA was poured onto the surface of sterile glass slides, which were suspended by sterile glass rods in sterilised petri dishes. The agar was allowed to solidify before isolates were streaked onto the glass slides that were then covered with sterile cover slips using sterile forceps. The petri-dishes were incubated for 24 hr at 30 °C. The slides were removed and examined microscopically using the oil immersion objective for pseudomycelium formation, which is indicated by filamentous growth of the isolates. A control was set up without streaking the isolate on it. Characterised yeasts were then identified according to Kreger van Rij (1984) and Barnett *et al.* (1990).

Mould isolates were characterised and identified according to their micro-morphology as well as the colour and morphology of their sporulating structures and conidia. Young, actively growing moulds were picked with a sterile needle onto clean glass slides and prepared for microscopic observation using lacto-phenol as mountant and cotton blue as stain (Barnett and Hunter, 1972; Harrigan and McCance, 1976). The slides were carefully covered with cover slips to exclude air bubbles. Microscopic examination of the prepared slides was carried out first using the low power objective followed by the x 40 objective lens for a closer examination of a selected field. Microscopic identification was on the basis of the structures bearing the spores and on the spores themselves. Other features observed included presence or absence of septation, rhizoid and other tissues (Onions *et al.*, 1981).

Antimicrobial activities of juice extracts. The antimicrobial activities of the juice extracts were tested against two bacterial pathogens (*Escherichia coli* and *Salmonella typhi*) and one pathogenic yeast species (*Candida albicans*). The cells of the test organisms were used for antimicrobial testing by seeding in appropriate agar plates at ca. $\log_{10}$ 7.0 CFU ml^{-1}.

The agar well diffusion method was used because it is known to allow better diffusion of the extracts into the medium thus enhancing contact with test organisms and ensuring more accurate results (Omenka and Osuoha, 2000).

Exactly 0.1 ml of 24 hr broth culture of each of the test microorganisms was aseptically seeded into nutrient agar plates for the bacteria and malt extract agar for yeast. The media were allowed to solidify before making the agar wells using a

sterile cork borer (5.0 mm diameter). A total of 0.5 ml of the test extracts were introduced into the wells before they were incubated at 37 °C for 24 hr for bacteria; 30 °C for 48 hr for yeast. The zones of inhibition were measured in millimeters.

Statistical analysis. Data generated from the study were analysed by one-way analysis of variance (ANOVA) at 5% level of significance and bivariate correlations using the Statistical Package for Socio-scientists (SPSS) version17.0 for Windows (SPSS Inc, Chicago, USA).

RESULTS AND DISCUSSION

Six bioactive compounds were identified from phytochemical analysis of the juice extracts of *Hibiscus sabdariffa* and *Syzygium samarangense* (Table 1). Analysis revealed the presence of alkaloids, tannins, saponins, flavonoids, phenols and glycosides. Saponins were the most abundant bioactive compounds present with *Syzygiumsamarangense* having a higher concentration (4.77%) than *Hibiscus sabdariffa* (1.46%). Flavonoids were, however, more in *Hibiscus sabdariffa* (2.41%) than in *Syzygium samarangense.*

The presence and effects of these biologically active compounds had been reported in related plants with potential medicinal properties (Iwu, 1993; Adeniyi *et al.*, 1996; Fasola, 2000). These chemical compounds are known to display inhibitory activities against many microorganisms (Rojas *et al.*, 1992; Banso and Olutimeyin, 2001; Onyilagha and Shahidul, 2009). Therefore, this study confirms and corroborates previous studies and affirms the potentials of these plants for pharmacological purposes.

Herbs that contain tannins are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003). Flavonoids and tannins are antioxidants that improve immune function, prevent heart diseases and some cancers (Trease and Evans, 1989). The ingestion of the aqueous solution of saponins is harmless to man (Babajide *et al.,* 1999). One of the largest groups of chemicals produced by plants are the alkaloids and their amazing effect on humans has led to the development of powerful pain killer medications (Raffauf, 1996). Bioactive compounds are constituents that are found in certain foods. A wealth of scientific literature from numerous types of epidemiological and case controlled studies have identified the potential relationships between bioactive compounds (or "functional" components) and their protective effects against hypertension, cardiovascular disease, cancer, and other health conditions.

From microbial enumeration using appropriate selective media, viable counts were in the order of 10^4 cfu ml^{-1} (Table 2). The highest counts were recorded on MacConkey and Nutrient Agar plates. This was probably due to contamination from handling. Microbiological analysis further revealed 10 genera of bacteria and four moulds in the juice extracts. Four organisms, *Bacillus cereus, Streptococcus faecium, Aspergillus tamarii* and *Eurotium repens,* were present in the two juice extracts (Table 3). *Bacillus cereus*

TABLE 1. Concentration of bioactive metabolites in extracts of *S. samarangense* and *H. sabdariffa*

Metabolites	*S. Samarangense*	*H. sabdariffa*
Saponins (%)	4.77 ± 0.02^{a}	1.46 ± 0.01^{b}
Alkaloids (%)	0.56 ± 0.01^{b}	0.08 ± 0.01^{e}
Tannins (%)	0.25 ± 0.01^{c}	0.18 ± 0.01^{c}
Total phenols (%)	0.09 ± 0.01^{d}	0.08 ± 0.01^{e}
Flavonoids (%)	0.56 ± 0.01^{b}	2.41 ± 0.02^{a}
Glycosides (%)	0.08 ± 0.01^{d}	0.13 ± 0.01^{d}

Values are means of three replicates ±Standard Deviation. Values with the same superscripts are not significantly different at 5% level of significance along the columns

TABLE 2. Total counts (cfu ml^{-1}) of microorganisms isolated from *S. samarangense* and *H.sabdariffa*

Enumeration media	*H. Sabdariffa*	*S. samarangense*
Plate count agar	$1.47 \times 10^{4\,b}$	$1.07 \times 10^{4\,c}$
Nutrient agar	$2.23 \times 10^{4\,a}$	$1.73 \times 10^{4\,a}$
Mac conkey agar	$2.80 \times 10^{2\,d}$	$2.67 \times 10^{2\,d}$
Potato dextrose agar	$1.20 \times 10^{4\,c}$	$1.10 \times 10^{4\,b}$
Mannitol salt agar	$1.0 \times 10^{1\,e}$	$0.8 \times 10^{1\,e}$

Values are means of three replicates ± Standard Deviation. Values with the same superscripts are not significantly different at 5% level of significance along the columns.

is a well known bacterial food-borne pathogen while *Aspergillus tamarii* and *Eurotium repens* are producers of mycotoxins, especially the carcinogenic aflatoxins (Bryan, 1988; Gachomo *et al.*, 1993).

From the study, *Escherichia coli* and *Candida albicans* had the greatest zones of inhibition against Roselle; while *Salmonella typhi* was the most sensitive to *S. Samarangense* (Table 4). The reasons for this observed trend could not be deduced from previous reports and can be noted as new information that will contribute to identifying the ranges of activities and modes of action for drugs that can be developed from Roselle and pink wax apple.

TABLE 3. List of isolated microorganisms from extracts of *S. samarangense* and *H. Sabdariffa*

Isolated organisms	*H. sabdariffa*	*S. samarangense*
Bacillus cereus	+	+
Bacillus firmus	+	-
Micrococcus acidophilus	-	+
Morganella morganii	-	+
Pseudomonas aeruginosa	+	-
Pseudomonas fragi	+	-
Proteus vulgaris	+	+
Serratia marcescens	+	-
Staphylococcus aureus	-	+
Streptococcus faecium	+	+
Aspergillus tamarii	+	+
Aspergillus niger	+	-
Aspergillus fumigatus	+	-
Eurotium repens	+	+

+ = isolate is present in juice; - = isolate is not present in juice sample

TABLE 4. Antimicrobial activities of *S. samarangense* and *H. sabdariffa* juice extracts

Test organisms	Zones of Inhibition (mm)	
	H. sabdariffa	*S. samarangense*
Escherichia coli	45.7 ± 0.1 [a]	32.8 ± 0.2 [c]
Salmonella typhi	43.7 ± 0.1 [b]	47.0 ± 0.2 [a]
Candida albicans	45.0 ± 0.4 [a]	32.0 ± 0.1 [c]
Chloramphenicol	41.0 ± 0.4 [c]	40.5 ± 0.4 [b]

Values are means of three replicates ± = Standard Deviation. Values with the same superscripts are not significantly different at 5% level of significance along the columns

CONCLUSION

In this study, *Hibiscus sabdariffa* and *Syzygium samarangense*juices showed significant antimicrobial properties against common human pathogens tested.The antimicrobial activities demonstrated by the extracts of Roselle and pink wax apples justify some of the ethno-pharmacological claims about these plants in the treatment of some common ailments. The bioactive constituents of these plants need to be isolated, purified and analysed for possible use in making drugs.

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