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Original Research Article

In vitro activity of Extract and Fractions of *Albizia chevalieri* Harms (Mimosaceae) Stem Bark against *Trypanosoma brucei brucei*

Ngozi B. Uzoka[1,2*], Chika J. Mbah[2], Charles O. Nnadi[2], Samuel E. Okhale[3] and Ngozi J. Nwodo[2]

[1]*Nigerian Institute for Trypanosomiasis Research (NITR), South East Zonal Office, Independence Layout 40007, Enugu State, Nigeria.*
[2]*Pharmaceutical and Medicinal Chemistry Department, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria*
[3]*National Institute for Pharmaceutical Research and Development (NIPRD), Idu Industrial District, 900106, Abuja, Nigeria.*

ABSTRACT

African trypanosomiasis is a parasitic infection that adversely affects man and livestock in sub-Saharan Africa. In addition to the lack of vaccines or drugs for prophylaxis, existing chemotherapies are toxic, expensive, and have developed resistance to trypanosomes. The study investigated the *in vitro* activity of *Albizia chevalieri* stem bark extract and fractions against *Trypanosoma brucei brucei*. The methanol extract was partitioned successively in graded solvents to obtain n-hexane, n-butanol, ethyl acetate and aqueous fractions. The phytochemical, acute toxicity, and *in vitro* anti-trypanosomal assays were conducted following the Harbone, Lorke and Drug Incubation Survival Test (DIST) methods respectively. *Albizia chevalieri* stem bark extract was found to be considerably safe, and rich in important bioactive phytoconstituents such as alkaloids, terpenoids, flavonoids and glycosides. The *in vitro* activity against *T. brucei brucei* showed that 0.3125 µg/mL of the extract, butanol fraction and diminazene aceturate standard caused 96.0 ± 2.51, 96.50 ± 1.70 and 93.40 ± 3.24 % inhibition of parasites motility respectively; corresponding to 1.00 ± 0.63, 0.83 ± 0.48 and 1.69 ± 0.82 trypanosomes/field. The strong inhibition of *T. brucei* motility by the extract and its butanol fraction has contributed significantly to knowledge as the first report of the anti-trypanosomal activity of *A. chevalieri* against *Trypanosoma brucei brucei*

Keywords: Anti-trypanosomal, *Albizia chevalieri*, Drug Incubation Survival Test, Phytochemical

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Introduction

African trypanosomiasis is caused by a protozoan parasite of the genus *Trypanosoma*, and transmitted by tsetse flies to both humans and animals. It adversely affects man and animals and constitutes one of the Neglected Tropical Diseases (NTDs) occurring in 37 endemic African countries. It causes African trypanosomiasis which may be human trypanosomiasis or sleeping sickness and animal trypanosomiasis or nagana affecting man and animals respectively. [1] Available epidemiological data show that sleeping sickness is predominant in twenty-four West and Central African countries where it is responsible for 98% of all reported cases while *Trypanosoma brucei rhodesiense* takes an acute course and is predominant in thirteen countries in both East and Southern Africa, where it is responsible for 2% of all reported cases. [2] Both forms of HAT could be fatal if left untreated. [3] *Trypanosoma. brucei* is restricted to sub-Saharan Africa due to the occurrence of the tsetse fly only in tropical Africa but worth mentioning, is the establishment of *T. vivax* in parts of Central and South America and the Caribbean, which are free of tsetse flies. [3]

*Corresponding author. Email: ngozi.uzoka@yahoo.com
Tel: +2348023757033

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Nagana affects mostly cattle among domestic animals and some susceptible wild animals. [4] With efforts towards control, the WHO NTD roadmap aimed at zero transmission (eradication) to humans by 2030. [6] Vector control and chemotherapy are approaches available for control and possible elimination of the disease. [7] These two approaches have not been as effective as expected due to undesirable environmental pollution arising from the use of insecticides, an increase in drug resistance by the parasites to existing drugs and a lack of vaccine. [8-10] Vaccine development has proven difficult and practically impossible due to the immune evasion mechanism of trypanosomes. The trypanosome surface is enveloped by a glycoprotein called the variant surface glycoprotein (VSG) which is the main antigenic determinant of the human immune system. The genome contains over a thousand genes that are capable of coding for the VSG genes which switch randomly at each generation. [11,12] More so, the currently available chemotherapy relies on only a few drugs; eflornithine used in combination with nifurtimox, which is limited by cost, accessibility, toxicity and cumbersome treatment regimen. It is well-known that medicinal plants have shown potential as a source of lead compounds in drug development and many medicinal plants have been reported to have shown anti-trypanosomal activities. [13]

Albizia chevalieri Harms is a flowering shrub used as a purgative, cough remedy, taenicidal, remedy for dysentery, cancer, and diabetes mellitus in ethnomedicine. [14,15] The plant also has documented pharmacological activities such as antibacterial, anti-inflammatory and antioxidant properties and anti-venom activities. [16] Several *Albizia* species including *Albizia schimperiana Albizia gummifera* and *Albizia zygia* possess anti-trypanosomal activities while *A. chevalieri* has shown strong potential in folk medicine. [17,18] This study, therefore, investigated this potential activity of the stem bark against *Trypanosoma brucei brucei* using an *in vitro* experimental model.

Materials and Methods

Instruments and reagents
The methanol, ethyl acetate, hexane and butanol were obtained from Molychem India, RPMI 1640 medium (Caisson Laboratory, USA) containing L-glutamine and sodium carbonate (for the *in vitro* assay). Gentamicin (40 µg/L), 10% (v/v) heat-inactivated goat serum and 1% (w/v) glucose (for media preparation). Phosphate buffered saline (PBS), diminazene aceturate (Berenil), rotary evaporator (RE-1002, China), weighing balance (AR 1530 analytical balance, US), water bath (Shanghai, China), oven (PL 402-L, Switzerland), vacuum pump (PL 402-L, Switzerland)., SHB series water circulating multi-purpose vacuum pump, water bath with shaker (Shanghai, China).

Experimental animals
Albino mice (23-26 g) were housed at the animal house of Veterinary Medicine, University of Nigeria. They were allowed to acclimatize for 7 days at the experimental site. They were kept in plastic cages at 25 ± 2 °C and relative humidity, with access to feed and water *ad libitum*. The ethical clearance was obtained from the University of Nigeria Animal Care and Use Committee (ACUC) protocol (Approval reference number: FVM-UNN-IACUC-2024-04/150).

Plant material
Albizia chevalieri stem bark was collected from Orba (6º 51' 0" North, 7º 27' 0" East), Enugu State, Nigeria in September 2023. The stem bark was identified, collected and authenticated by Mr. Alfred Ozioko, a taxonomist with the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria. The voucher specimen was deposited at the herbarium of the Centre with voucher number INTERCEDD/16316.

Trypanosoma brucei parasite and culture
Trypanosoma brucei brucei bloodstream forms were obtained from the Veterinary Parasitology unit of the University of Nigeria, Nsukka. The parasite was cultured through serial passage in albino rat donors every 48 h

Extraction
Dried and pulverised stem bark (2 kg) was cold macerated in 10 L of methanol (95% v/v) for 48 h and agitated at intervals. [19] After 48 h, the mixture was filtered using a muslin cloth and then re-filtered using Whatman filter paper. The marc was further macerated in fresh 5 L of methanol for another 24 h with intermittent agitation. Thereafter, the mixture was filtered using muslin cloth and Whatman filter paper respectively. The combined filtrate was concentrated using a rotary at 40 °C under reduced pressure and further left to air-dry in the laboratory to produce a constant weight of 260.46 g of a dark brown extract (AME).

Solvent partitioning of extract
The extract (AME), 120 g was dispersed in 500 mL of 10% v/v aqueous methanol to form a uniform dispersion, successively partitioned with *n*-hexane (500 mL x 2), ethyl acetate (500 mL x 2), *n*-butanol (500 mL x 5) and the aqueous phase based on the polarity of solvents using a 2000 mL separatory funnel. [20] The fractions were concentrated using a rotary evaporator under reduced pressure and placed in a desiccator to a constant weight to afford *n*-hexane (AHF), ethyl acetate (AEF), butanol (ABF) and aqueous (AAQ) fractions respectively. The extract and fractions obtained were assayed for anti-trypanosomal activity.

Phytochemical screening
Phytochemical screening of the extract and fractions was carried out according to standard methods. [21]

Acute toxicity study of extract
An acute toxicity test was performed according to Lorke protocol. [22] The first stage involved nine mice divided into groups A, B and C, and administered 10, 100, 1000 mg/kg of the extract to the groups respectively. With no death recorded after 24 h, the second stage of the test was carried out. Three doses of the plant extract were administered (1600, 2900, 5000 mg/kg) and observations were made for mortality up to 24 h.

Collection of parasitized blood
Blood samples containing *T. brucei brucei* were collected from donor rats in a 5 mL syringe using the cardiac puncture technique under chloroform anaesthesia. The samples were then dispensed into an EDTA container and gently mixed to prevent clotting

Preparation of samples for in vitro assay
Solutions of the extract, fractions and standard drug (diminazene aceturate) were reconstituted in supplemented dimethyl sulfoxide. A 20 mg/mL stock solution of the respective extract, fractions and standard drug was prepared. Serial dilutions to yield working solutions with concentrations of 10 to 0.15625 mg/mL were made from their respective stock solution.

In vitro assay of extract and fractions
The Drug Incubation Survival Test (DIST) was employed for the *in vitro* antitrypanosomal assay. Reconstituted solutions of each extract, fractions, and the reference drug (DA) were dispensed in triplicates (100 µL) into the wells of a 96-well microtiter plate. A 20 µL blood suspension containing *T. b. brucei* was then added to each well and gently mixed. Control wells included 100 µL of supplemented medium and 20 µL of blood suspension only. The microplate was placed in a desiccator with approximately 5% carbon dioxide and incubated at 37 °C. Wet smears were prepared 6 h post-incubation from each well. Each smear was examined under a light microscope (400× magnification), with trypanosome counts taken from three fields of view across nine observations per sample concentration.[4] Trypanosome counts from control well smears were similarly recorded. A reduction in the number of motile trypanosomes compared to control wells served as an index for *in vitro* activity

Determination of parasitemia
To estimate the trypanosome count in the blood smear of infected rats, the rapid matching method developed by Herbert and Lumsden (1981) was employed.[9] A drop of whole blood was collected from each rat via tail snip and immediately applied to a clean, grease-free glass slide. A cover slip was then carefully placed over the drop to create a thin, even circular film of blood. The prepared slide was examined under a light microscope (Image Solutions, Preston, UK) at a magnification of 400×. Using Lumsden's chart as a reference, the distribution of trypanosomes within the red blood cells (RBCs) was analysed. The number of trypanosomes observed was then matched against the chart to estimate the approximate concentration of trypanosomes per millilitre of blood. This rapid matching method provided a reliable measure of parasitemia in the infected rats

Statistical analysis
Data obtained were analysed statistically. The variance between mean values was evaluated using a one-way Analysis of Variance (ANOVA), with a significance level of $p < 0.05$ and Duncan's multiple range test was performed for post-hoc comparisons.

Results and Discussion

African trypanosomiasis is a public health concern in sub-Saharan. World Health Organisation has increased interest in using traditional medicine over the years since it can be safe, effective, cost-effective, and easy access to treatment. [2,4-7] Despite the recent development of fexinidazole, it is still important to continue with the search for new trypanocides from natural sources due to fear of parasite resistance to fexinidazole. [9-11] Investigating the anti-trypanosomal activity of *A. chevalieri* stem bark on *T. b. brucei* was the goal of this study to complement the taenicidal, antidiabetic, antimicrobial, anti-cancer, anti-inflammatory, anti-oxidant and anti-venom activities of *A. chevalieri* which may help develop novel trypanocides. [14-18]

Extraction of plant material
A 2000 g of dried coarse sample of *A. chevalieri* stem bark extracted with methanol (95%v/v) yielded 260.5 g of dark brown extract

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designated as AME, representing 13.0% of dry weight of coarse powder. On subsequent partitioning in solvents of increasing polarity, 120.0 g of the methanolic crude extract yielded (AHF, 12.6 g), (AEF, 16.16 g), (ABF, 47.82 g) and (AAQ, 28.61 g) representing 1.37, 1.75, 5.19 and 3.10 %w/w relative to dry weight of coarse powder of *n*-hexane, ethyl acetate, and *n*-butanol and aqueous soluble fractions respectively. Extraction methods and extracting solvents play significant roles in bioprospecting for trypanocidal compounds. [23] The choice of extraction method and fractionation solvents also affect the yield and nature of secondary metabolites from natural sources due to the relative polarity differences of secondary metabolic products of plants. In this study, the high yield of *n*-butanol could be a result of the higher polar compounds in the extract which was also evident in the abundance of alkaloids, glycosides and flavonoids in the *n*-butanol fraction (Table 1). The significantly high yield of methanol (13%w/v) on cold-maceration and solvent fractions of *n*-butanol (5.19%w/v) and aqueous (3.10%w/v) indicated an abundance of polar constituents.

Table 1: Qualitative phytochemical composition of *A. chevialeri*

Phytoconstituents	AME	AHF	AEA	ABF	AAQ
Saponins	+	-	-	+	+
Tannins	+	-	-	-	+
Cardiac Glycosides	+	-	+	+	+
Flavonoids	+	+	+	+	+
Terpenes and Sterols	+	+	+	-	_
Alkaloids	+	+	+	+	+
Anthraquinone	+	+	+	_	_

Key: (+) = Present, (-) = Absent; *A. chevialeri* methanol extract (AME), *A. chevialeri n*-hexane fraction (AHF), *A. chevialeri* ethyl acetate fraction (AEF), *A. chevialeri* butanol (ABF) fraction and *A. chevialeri* aqueous (AAQ) fraction

Phytochemical analysis

The extract showed a rich presence of flavonoids, terpenoids, saponins, tannins, and cardiac glycosides. Tannins were absent in all the fractions except AAQ, there was the presence of flavonoids and alkaloids in all the fractions while saponins were only present in ABF and AAQ; anthraquinone, terpenes and sterols were also absent in the two fractions. Cardiac glycosides were absent in AHF but present in other fractions. Phytochemical analysis of the extract indicated it is rich in phytoconstituents of pharmacological importance; saponins, tannins, cardiac glycosides, flavonoids, terpenes, sterols, alkaloids and anthraquinone. Carbohydrates were also present. Phytoconstituents such as flavonoids, alkaloids, terpenes, quinones, polyphenols, triterpenoids and sterols are the most frequently reported phytochemicals with anti-trypanosome activities. [13] Similarly, the presence of alkaloids, terpenoids, polyphenols and flavonoids in this study tend to agree with other reports from previous studies which showed that most compounds isolated from plants with activities against protozoan and parasitic diseases contained alkaloids, polyphenols, terpenoids and flavonoids. [6,13] Documented evidence shows that plants with flavonoids and alkaloids have anti-trypanosomal activity. Anti-trypanosomal activities of alkaloids have been associated with intercalation of DNA, induction of apoptosis, inhibition of microtubule assembly as well as protein synthesis as a mechanism of their action against trypanosomiasis. [13] A reported anti-trypanosomal activity exhibited by the presence of saponins, steroids and terpenoids while a combination of alkaloids with steroidal saponins showed synergistic potentiation of trypanocidal activity. [13] Similarly, triterpenoids and sterols have been documented to possess anti-trypanosomal activity. [6]

Acute toxicity assay

There was no mortality in the mice during or after the test. Animals did not show any form of discomfort including scratching the tongue, abnormal or restlessness. There was no treatment-related toxicity. The extract was relatively safe with LD_{50} above 5000 mg/kg. Following the acute toxicity test, no mortality nor sign of toxicity was recorded. The acute toxicity of *A. chevalieri* indicates that the plant part used is considerably safe having a median lethal dose, LD_{50} estimated to be above 5,000 mg/kg. This corresponds with reports that extract with LD_{50} value of 5000-15,000 mg/kg body weight showed that the extract is practically non-toxic. [24]

In vitro antitrypanosomal assay

After 6-h post-incubation, the extract, fractions and standard drug, diminazene aceturate, showed a dose-dependent antitrypanosomal activity. At a concentration of 0.3125 mg/mL, parasite motility was significantly reduced ($p < 0.05$) to 0.83 ± 0.48 by ABF compared to the standard drug, diminazene aceturate, 1.67 ± 0.82 (Table 2). The extract, fractions and diminazene aceturate inhibited parasite motility at concentrations of 10 and 5 mg/mL, while at 2.5 mg/mL, motility ceased for all test agents except for AHF which had 9.67 ±1.50 parasite count per field compared to the untreated 24.3 ± 1.63.

Table 2: Number of surviving trypanosomes six-hour post-incubation

Conc. (mg/mL)	Trypanosome counts/field					
	AME	AHF	AEF	AAQ	ABF	Diminazene
10	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
5	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
2.5	0.00±0.00^{a}	9.67±1.50^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
1.25	0.00±0.00^{a}	13.3±1.75^{c}	0.33±0.52^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
0.625	0.33±0.52^{ab}	14.2±1.83^{cd}	4.67±1.51^{b}	1.67±1.21^{b}	0.00±0.00^{a}	0.00±0.00^{a}
0.3125	1.00±0.63^{b}	15.7±2.34^{d}	8.67±1.75^{c}	11.5±1.21^{c}	0.83±0.48^{a}	1.67±0.82^{b}
0.15625	9.67±1.63^{c}	19.2±1.83^{e}	13.8±2.22^{d}	15.7±1.03^{d}	8.50±2.42^{b}	3.50±0.84^{c}
Control	25.2±0.98^{d}	24.3±1.63^{f}	24.3±1.63^{e}	24.0±1.67^{e}	25.2±0.98^{d}	24.0±1.67^{c}

Values are given as mean ± standard deviation; in each column, values with different superscripts between rows have statistically significant differences ($p < 0.05$); *A. chevialeri* methanol extract (AME), *A. chevialeri n*-hexane fraction (AHF), *A. chevialeri* ethyl acetate fraction (AEF), *A. chevialeri* butanol (ABF) fraction and *A. chevialeri* aqueous (AAQ) fraction

Similarly, at 1.25 mg/mL of AME, AAQ, ABF and diminazene aceturate, parasite motility was completely inhibited but there was still trypanosome motility in the culture treated with AHF and AEF. However, at 0.625 mg/mL, there was complete cessation with ABF and diminazene aceturate while AME, AHF, AEF and AAQ recorded reduced parasite counts per field of 0.33 ± 0.52, 14.2 ± 1.83, 4.67 ±1.51, and 1.67 ± 1.21 mg/mL respectively when compared to their respective control. At treatment concentrations ≤ 0.3125 mg/mL, significant parasite motility was recorded. Anti-trypanosomal activities of saponins have also. The activity of acylated triterpenoid saponins previously isolated from *A. chevalieri* stem bark exhibited pro-apoptotic activity, likewise, triterpenoid saponins consisting of from Albizia genus demonstrated inhibitory activity of the growth of many cancer cells line *in vitro* model shows that there could be a positive relationship which exists between anti-tumour properties and anti-trypanosomal properties. [25] The presence of pharmacologically significant phytoconstituents present in the extract and active fraction may have acted singly or in synergy.

The inhibition of trypanosome motility by the extract and fractions of *A. chevialeri* is shown in Table 3. At concentrations of 10, 5 and 2.5 mg/mL, the extract, fractions indicated 100% inhibition of parasites except for AHF which had about 60% inhibition at 2.5 mg/mL. AME, AAQ, ABF and diminazene aceturate indicated 100% inhibition at 1.25 mg/mL while AHF and AEF had 45.2 ± 7.19 and 98.6 ± 2.12. ABF and diminazene aceturate had 100% inhibition up to 0.625 mg/mL with the notable activity of 96.5 ± 1.70 % and 93.4 ± 3.24 % respectively at a low concentration of 0.3125 mg/mL with *p*-value, < 0.05.

Table 3: Inhibition of trypanosome motility

Conc. (mg/mL)	*T. b. brucei* motility inhibition (%)					
	AME	AHF	AEF	AAQ	ABF	Diminazene
10	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00
5	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00
2.5	100.0± 0.00	60.3±6 .19	100.0± 0.00	100.0± 0.00	100.0± 0.00	100.0± 0.00
1.25	100.0± 0.00	45.2±7 .19	98.6±2 .12	100.0± 0.00	100.0± 0.00	100.0± 0.00
0.625	98.7±2 .05	41.8±7 .54	80.8±6 .18	93.1±5 .05	100.0± 0.00	100.0± 0.00
0.3125	96.0±2 .51	35.6±9 .60	64.4±7 .19	52.1±4 .37	96.5±1 .70	93.4±3 .24
0.15625	61.6±6 .49	21.2±7 .54	43.2±9 .16	34.7±4 .30	64.6±1 0.1	86.1±3 .32

Values are given as mean ± standard deviation; *A. chevialeri* methanol extract (AME), *A. chevialeri n*-hexane fraction (AHF), *A. chevialeri* ethyl acetate fraction (AEF), *A. chevialeri* butanol (ABF) fraction and *A. chevialeri* aqueous (AAQ) fraction

In this study, parasite motility was considered a relatively reliable indicator of the viability of trypanosomes. [26] Lack of parasite motility in the study compared to the control was taken as the death of the parasite likewise, parasite motility was considered as survival of the trypanosomes hence decrease in the number of surviving trypanosomes was taken as a measure of the anti-trypanosomal activity. [26] The number of surviving trypanosomes decreased with increase in the concentration of the extract, fractions and diminazene aceturate. As observed from the results, the number of surviving trypanosomes at a concentration range of 0.15625 mg/mL to 10 mg/mL decreased, and there was complete cessation of parasite motility 0.00 ± 0.00 at a concentration of 0.625 mg/mL on administration of ABF and diminazene aceturate compared to their respective control groups. Medicinal plants serve as a rich source of lead compounds for chemotherapy development. It is important to continue with the search and identification of active compounds with activities against African trypanosomiasis which has been challenging for rural sub-Saharan Africa, to ensure access to affordable, safe, and effective trypanocides.

In this study, therefore, it is imperative to state that the phytoconstituents or the concentration of the bioactive compound(s) was unknown. However, several phytoconstituents have been implicated in the anti-trypanosomal activity of plants. [27] The significantly higher activities of the extract and its *n*-butanol fraction on fractionation suggest that alkaloids, saponins, cardiac glycosides and/or flavonoids content of *A. chevalieri* could be the major active phytoconstituents. To confirm the link with the present study, however, would require further separation of the fraction, isolation of the bioactive compound(s) from the n-butanol fractions; which is currently in progress and quantification of the isolated trypanocides.

Conclusion

The extract and *n*-butanol fractions of *Albizia chevalieri* showed significant anti-trypanosomal activity by inhibition of motility and clearance of trypanosomes from the bloodstream of the experimental animals. The extract contained biologically active secondary metabolite and also showed no sign of acute toxicity. This study has contributed to knowledge as the first report of the anti-trypanosomal activity of *A. chevalieri* against *Trypanosoma brucei brucei*

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original. Any liability for claims relating to this article will be borne by us.

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Author Contributions

Conceptualization and supervision: Ngozi Nwodo and Chika Mbah; formal analysis: Ngozi Uzoka and Charles Nnadi; Methodology: Ngozi Uzoka and Samuel Okhale; Writing manuscript draft, review and editing: Ngozi Uzoka, Ngozi Nwodo and Charles Nnadi

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