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In vitro toxicity, anti-inflammatory and antioxidant activities of ethanolic root extracts of Tanzanian *Hydnora abyssinica* A. Br. (Aristolochiaceae)

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Injuries and the effects of diseases, including various types of cancer, are among the sources of inflammation, pain, and discomfort for patients. *Hydnora abyssinica*, claimed by traditional practitioners in the Eastern Same district to manage inflammation, may contain ingredients with anti-inflammatory activity. This study aimed to evaluate the toxicity and determine the anti-inflammatory and antioxidant activities of the ethanolic extract of the plant's rhizomes. Anti-inflammatory activity was assessed through the inhibition of protein denaturation, and antioxidant activity was evaluated using the phosphomolybdenum method (total antioxidant capacity assay, TAC) with a UV/Vis spectrometer at wavelengths of 660 and 695 nm, respectively. Toxicity was determined by the Brine shrimp lethality test (BST). The extract was tested for anti-inflammatory activity compared to the control, diclofenac sodium, and antioxidant activity was assessed compared to the standard, ascorbic acid. Toxicity was compared to cyclophosphamide, a known toxic anticancer agent. The extract's toxicity was found to have an LC_{50} of 48.0286 µg/ml and a 95% CI of 33.0973-69.6973. In contrast, cyclophosphamide's toxicity had an LC_{50} of 15.0865 µg/ml and a CI of 7.6196-29.8717. The extract revealed anti-inflammatory activity with an IC_{50} of 279.1 ± 1.124 µg/ml, which is more active than the control, diclofenac sodium, with an IC_{50} of 537.2 ± 0.5643 µg/ml. The ethanolic extract had a total antioxidant capacity of 8.1893 ± 0.0037/g AAE. Active ingredients from *H. abyssinica* may serve as substitutes for diclofenac sodium, ibuprofen, and other anti-inflammatory agents with equal or less activity.

Key words: *Hydnora abyssinica*, anti-inflammatory, protein denaturation, antioxidant, toxicity.

INTRODUCTION

Natural products have a long history of serving as a source of food and medicinal agents, with plants being the primary source of most drugs (Cragg and Newman, 2013). Despite this fact, only a few drugs are processed and commercially applied, whereas around 80% of the world's population, particularly in developing countries, relies on medicinal plants for healthcare, as prepared by traditional practitioners (Hishe et al., 2016). The search

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for medicinal plants used by traditional healers not only aims at drug discovery but also seeks to identify plants threatened by climate change, human activities, and excessive harvesting (Anand et al., 2019). *Hydnora abyssinica* A. Br. is one of the species in the genus *Hydnora*, which includes *Hydnora africana*, *Hydnora johannis*, *Hydnora esculenta*, and *Hydnora triceps*, rarely collected by botanists (Mkala et al., 2021; Al-Fatimi et al., 2016; Williams et al., 2011a). This plant is challenging to locate due to its subterranean growth habit, producing seasonal flowers that remain aerial for a short time (Williams et al., 2011b). During the dry season, when flowers are absent, locating the plant is difficult without prior knowledge of its location. The plant's body is a corrugated rhizome with dark brown root bark and pinkish interior flesh; local people in the Same community refer to it as "Kiviža" in Pare (Ibrahim and Ibrahim, 1998). The rhizome of *H. abyssinica* is traded and traditionally used in East and Southern Africa to treat various ailments, including diarrhea, piles, acne, menstrual problems, stomach cramps, and bleeding (Williams et al., 2011a). Traditional practitioners in Same-Kilimanjaro, Tanzania, use a concoction of the plant's rhizome to treat inflammation, arthritis, cancer, tumors, and tonsils. In Eastern Ethiopia, it is used to treat cancer, tumors, and inflammation, while in Kenya, it was traditionally used to treat infections, cancer, and retained placenta removal. The Maasai community in Arusha, Tanzania, uses *H. abyssinica* to treat pneumonia (Bussa and Belayneh, 2019; Onyancha et al., 2015).

These examples demonstrate that *H. abyssinica* is a medicinal plant traditionally used by various communities to manage health problems. Despite its medicinal value, there is limited knowledge about its short-term or long-term health effects on humans and animals. Scientific investigation is necessary to verify the claims made by traditional healers in the Same District, ensure safety, and provide guidance on its optimal use. Furthermore, assessing safety and validating claims requires determining antioxidant activity, as it contributes to the removal of metabolic by-products like Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Melo et al., 2015), which are known to induce certain non-communicable diseases (Wafula et al., 2017). This study aims to evaluate the toxicity, antioxidant activity, and anti-inflammatory activity of the ethanolic extract of *H. abyssinica* rhizomes, traditionally used by practitioners in Gonja and Mamba-Same Kilimanjaro to treat inflammation, tumors, cancer, and swellings.

Inflammation

Inflammation is a defensive response designed to eliminate invaders or facilitate repair of injured tissue (Brazil et al., 2019). Common causes of inflammation include non-communicable diseases such as cancer, arthritis, injuries, pathogen infections, and insect stings (Trinchieri, 2012).

While inflammation serves as a protective mechanism, severe or excessive inflammation can become more painful and debilitating than the initial cause. Acute inflammation is characterized by vasodilation, fluid exudation, and neutrophil infiltration. However, excessive inflammation can lead to tissue damage, physiological decompensation, organ dysfunction, and even death if severe (Arroyo et al., 2016). The inflammatory response has garnered renewed interest in recent years, as many diseases, such as rheumatic and allergic disorders, involve tissue damage resulting from the inflammatory response itself (Schett and Neurath, 2018). In these cases, the normally protective role of inflammation becomes detrimental when the response becomes excessive in magnitude or duration. Anaphylaxis and septic shock are two notorious examples of excessive and potentially lethal inflammatory responses (Okin and Medzhitov, 2012). Inflammation is a complex process frequently associated with pain, involving occurrences such as increased vascular permeability, protein denaturation, and membrane alteration (Gusev and Zhuravleva, 2022).

Anti-oxidants

An antioxidant is a substance capable of inhibiting oxidation, referring to any substance that, at low concentrations, significantly delays or prevents the oxidation of a substrate (Gupta, 2015). Metabolic processes in the human body produce ROS and RNS (Ifeanyi, 2018). These sub-toxic ROS and RNS can lead to alterations in cellular and extracellular redox states, because signal changes in cell functions, and contribute to disease formation, such as cancer (Shlapakova et al., 2020). Antioxidants are necessary to scavenge and prevent the formation of ROS and RNS (Fubini and Hubbard, 2003), thereby protecting the human body from damage caused by ROS and RNS (Bratovcic, 2020; Kozlov et al., 2024). The role of antioxidant substances in combating ROS and RNS makes the assessment of antioxidant activity a crucial test when searching for drugs to control non-communicable diseases.

Brine shrimps lethality test (BST)

Screening for toxicity is a mandatory step when searching for any drug intended for human or animal use to control diseases. Toxic drugs can damage both diseased and healthy cells, creating additional problems for patients. Before using a new drug, its toxicity level must be established through various tests, which may include animal models, cell lines, or organisms like *Artemia salina* L. (1758). The Brine Shrimp Test, first introduced by Meyers et al. (1982) and modified by subsequent researchers (Ntungwe et al., 2020), is a widely adopted method with minor modifications. This

test is simple, easy to apply, and uses standard, low-cost apparatus and reagents. In this study, the Brine Shrimp Lethality Test was employed to evaluate the toxicity of ethanolic extracts from the rhizomes of *H. abyssinica.*

MATERIALS AND METHODS

Plant

The rhizomes of *H. abyssinica* were collected with the assistance of a traditional practitioner and village leadership from Gonja Maore, located on the periphery of the Mkomazi Game Reserve in the Same District of Kilimanjaro.

Equipment, chemicals and reagents

Artificial seawater was collected from the seashore of Ocean Road. Chemicals, including Dimethyl Sulfoxide (DMSO), sulphuric acid, sodium phosphate, and others, were obtained from Sigma in Poole, Dorset, UK. Distilled water was also used. The test sample of plant extract was prepared, and equipment such as a hatching tank, measuring cylinder (1000 ml), spatula, and brine shrimp eggs from Aquaculture Innovations in South Africa were utilized. Additional equipment included an analytical balance, Pasteur pipette, light source (Table electric bulb), test tubes (12 × 100 mm), magnifying glass, and various pipettes as well as 10 ml flat round base vials, rubber sucker, lab. hot plate, 500 and 1000 ml beaker, 5 L plastic container, cotton wool, watch glasses, and a black background. Other materials used were 80% ethanol, egg albumin from fresh hen's egg, phosphate buffered saline (PBS) with a pH of 6.4, and injectable diclofenac sodium from Reyoung Pharmaceutical Co. Ltd in China. Cyclophosphamide from Loba Cheme and phosphate buffer from Chem Lab were also employed. The study utilized a UV/VIS spectrometer (Lambda 9000 Perkin Elmer) and an Agilent Gas Chromatography Mass spectrometer (GC-MS/MS) for analysis.

Sample preparation

The collected samples were packed in bags and transported to the Government Chemist laboratory in Dar es Salaam for analysis. Upon arrival, the samples were washed to remove soil and peeled to remove dead rhizome bark. The clean, brick-red rhizomes were sliced into small pieces and dried, taking care to avoid direct sunlight. Approximately 5 kg of dried samples were ground into a powder and sieved through a 2 mm sieve. One kilogram of the powdered sample (2 mm particles) was macerated in ethanol at a ratio of 1:3 w/v and left to soak for 24 h. The mixture was then filtered through filter paper, and the filtrate was subjected to rotary evaporation to remove the solvent. The resulting extract was collected, dried, and stored in the refrigerator for further analysis. Portions of the recovered extract were tested for toxicity, anti-inflammatory, and antioxidant activities.

Brine shrimps test

The test was performed according to the method developed by Meyers and modified by various researchers, including Mentor R (Hamidi et al., 2014). A stock solution of the extract (40 mg/ml) was prepared in DMSO and diluted to varying concentrations. A solution of 0.6% DMSO in artificial seawater served as the negative control. Ten brine shrimp larvae were introduced into each vial containing 5 ml of the test solution or control, in triplicate for each concentration level. After 24 h, the nauplii were examined against a lighted background, and the number of live larvae was counted. The mean percentage mortality was plotted against the logarithm of concentrations using GraphPad Prism version 5.1 software, which also provided regression equations, mean, standard deviation, and 95% confidence intervals (95% CI).

Antioxidant activity

The total antioxidant capacity was determined colorimetrically by the formation of a green color through the reduction of phosphomolybdate in the presence of phytoconstituents under acidic conditions, according to Prieto et al. (1999) with slight modifications. Exactly 500 µL of extract at different concentrations (10-200 µg/ml) was mixed with 3 ml of a reaction mixture containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate in a 1:1:1 ratio in test tubes. The test tubes were incubated at 95°C for 10 min to complete the reaction. After cooling to room temperature, the absorbance was measured at 695 nm using a UV/VIS spectrophotometer against a blank. Ascorbic acid was used as a reference standard. A calibration standard curve using ascorbic acid was plotted, and the antioxidant activity of the analytes was expressed and reported as the number of gram equivalents of ascorbic acid.

Anti-inflammatory activity

The ethanol extract (sample) and Diclofenac sodium (control) were prepared in concentrations ranging from 40 to 2500 µg/ml and analyzed in parallel. A reaction mixture consisting of 5 of 0.2 ml freshly prepared egg albumin (from fresh hen's egg) added to 2.8 ml of phosphate buffered saline (PBS, pH 6.4) was used. 2 ml of varying concentrations of the analyte/standard were added to tubes and gently mixed with the protein matrix to obtain final concentrations in a homogeneous mixture. Double-distilled water served as the negative control. The mixtures were incubated at 37 ± 2°C in a water bath for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm, using distilled water as the blank and mixed reagents without the analyte/standard as the sample blank. Diclofenac sodium, at final concentrations of 39.0625, 78.125, 156.25, 312.5, 625, 1250, and 2500 µg/ml, was used as the reference drug and treated similarly for determination of absorbance. The extract/drug concentration for 50% inhibition (IC_{50}) was determined by plotting the percentage inhibition with respect to the control against the treatment concentration (Mohan, 2021; Heendeniya et al., 2018; Chandra et al., 2012).

Identification of Compounds

The ethanolic extract of Tanzanian *H. abyssinica* was re-dissolved in ethyl acetate, filtered, and injected into an Agilent GC-MS/MS system, comprising an Agilent 7890B Series gas chromatograph and an Agilent 7693A autosampler. The carrier gas used was Helium at a flow rate of 1.2 ml/min, with an inlet pressure of 18.420 psi in constant flow mode. The injection mode was splitless, with an inlet temperature of 280°C and a wool single taper inlet liner. The injection volume was 1 µL. The oven temperature program started at 70°C for 0.5 min, then increased to 180°C at 25°C/min for 1 min, followed by a 6°C/min increase to 280°C for 8 min. The analytical column used was an Agilent J&W HP-5ms UI, 30 m × 0.25 mm × 0.25 µm. The mass selective detector was an Agilent 7000D Series with a performance turbo pump, operating in electron impact ionization mode with a transfer line temperature of 280°C and a source temperature of 250°C. The MS1 and MS2 quadrupole temperature was 150°C, with a solvent delay of 3 min a nd collision

gases of Helium and Nitrogen. Compounds were searched in the installed libraries, including W11N17mainLib (Willey, 2011; NIST, 2017).

Statistical analysis

Statistical analysis of the samples and control was performed in two separate experiments, each conducted in triplicate, resulting in two average observations for each concentration level. The final values were expressed as Mean ± SD (n=6). Data analysis was performed using GraphPad Prism version 5.1. The significance of the data in determining anti-inflammatory activity was assessed using a non-linear fit, with an R^2 value of 0.997. Meanwhile, the significance of the data in determining antioxidant activity was assessed using linear regression, with R^2 value of 0.9980 and $P < 0.0001$.

RESULTS AND DISCUSSION

Toxicity

The toxicity of the ethanolic extract of *H. abyssinica* rhizomes was determined to be 48.0286 µg/ml, with a 95% confidence interval (CI) of 33.0973-69.6973. In contrast, the control, cyclophosphamide, showed a toxicity of 15.0865 µg/ml, with a CI of 7.6196-29.8717. This is consistent with previously reported literature values for cyclophosphamide, which range around 16.3 µg/ml (Moshi et al., 2010). According to the United States National Cancer Institute (NCI), the toxicity to brine shrimp can be categorized as follows: highly cytotoxic (IC_{50} ≤ 20 µg/ml), moderately cytotoxic (IC_{50} = 21-200 µg/ml), weakly cytotoxic (IC_{50} = 201-500 µg/ml), and non-cytotoxic (IC_{50} > 501 µg/ml) (Niksic et al., 2021). All experimental results were expressed as mean ± SD of triplets. Based on these findings, the ethanolic extract of *H. abyssinica* rhizomes can be classified as moderately cytotoxic.

Compounds in rhizomes of *H. abyssinica*

The ethanolic extract of the rhizomes of *H. abyssinica* was analyzed using GC-MS (Agilent 7890B/7000D TQMS), which identified the presence of 12 phytochemical compounds. These compounds are listed as follows: butylated hydroxytoluene, hordenine, 2-propenoic acid, 3-(4-hydroxyphenyl)-methyl ester ($C_{10}H_{10}O_3$), 2-2-methylenebis(6(1,1-dimethyl)-4-methyl), anthraquinone, 3-(4-tert-butylphenyl)furan-2,5-dione, phenol, 4-ethyl-2-methoxy, lenthionine, naphthol-(1,2-6)thiophene, and 3-(4-tert-butylphenyl)furan-2,5-dione.

Antioxidant activity

The total antioxidant capacity of the ethanolic extracts of *H. abyssinica* and standard ascorbic acid was evaluated using the phosphomolybdenum method. Calibration curves were plotted using GraphPad Prism version 5.1, with triplets of concentration levels analyzed. The equation for the ascorbic acid plot was $y = 0.02353x - 0.019851$ ($R^2 = 0.9971$), while the equation for the *H. abyssinica* extract plot was $y = 0.002879x + 0.05565$ ($R^2 = 0.9980$). The total antioxidant capacity of the test samples was compared to that of the standard ascorbic acid using their respective equations, as shown graphically in Figures 1 and 2. The ethanolic extract of *H. abyssinica* revealed antioxidant activity with an IC_{50} value of 17,386.4709 ± 0.0037 µg/ml, while the activity of ascorbic acid showed an IC_{50} value of 2,125.7905 ± 0.0006 µg/ml. These results indicate that the antioxidant activity of the ethanolic extract of *H. abyssinica* is 8.179/g AAE, which is approximately eight times less active than ascorbic acid.

Anti-inflammatory activity

Researchers have long sought efficient, reliable, and affordable methods for testing anti-inflammatory activity. Various techniques have been employed, with varying degrees of success. While some scientists have used animal models, such as the foot oedema test in rats (Fierascu et al., 2018), others have opted for alternative methods like the cyclo-oxygenase bioassay (Shale et al., 1999; Moteetee and Kose, 2016) or enzymatic *in vitro* assays using isolated dairy product cells (Biswas et al., 2013).

However, monitoring protein denaturation has emerged as a promising approach for determining anti-inflammatory activity with minimal cost and without the need for experimental animals (Mohan, 2021; Heendeniya et al., 2018; Chandra et al., 2012). This method leverages the ability of non-steroidal anti-inflammatory drugs to stabilize heat-treated albumin at physiological pH (6.2-6.5) (Samaraweera et al., 2023; Johnson and James, 2022).

Protein denaturation is a well-documented consequence of inflammation, particularly in conditions like arthritis (Umapathy et al., 2010). By using this method, researchers can validate the anti-inflammatory activity of plant extracts and provide evidence for their traditional uses, paving the way for further exploration and potential applications.

Protein denaturation can serve as a reliable indicator of inflammation and offers a viable alternative to animal testing (Shah et al., 2017). In this study, the protein denaturation bioassay using fresh hen egg albumin was employed for the *in vitro* assessment of the anti-inflammatory properties of the ethanolic extract of *H. abyssinica* (Figures 3 and 4).

The results showed that both the extract and the reference drug, diclofenac sodium, inhibited heat-induced protein (albumin) denaturation, as evident from the increments in absorbance (Modak et al., 2017). Notably, the IC_{50} values revealed that the ethanolic extract of *H. abyssinica* was more effective than diclofenac sodium.

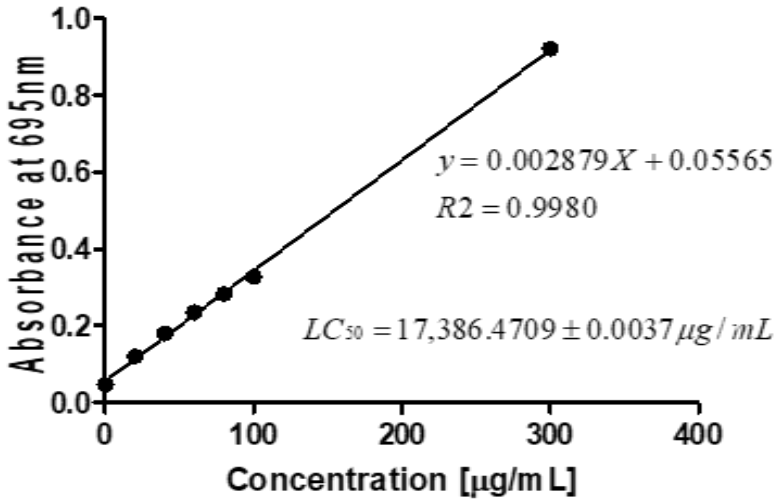

Figure 1. Antioxidant activity of ethanolic extract of Tanzanian *H. abyssinica.*

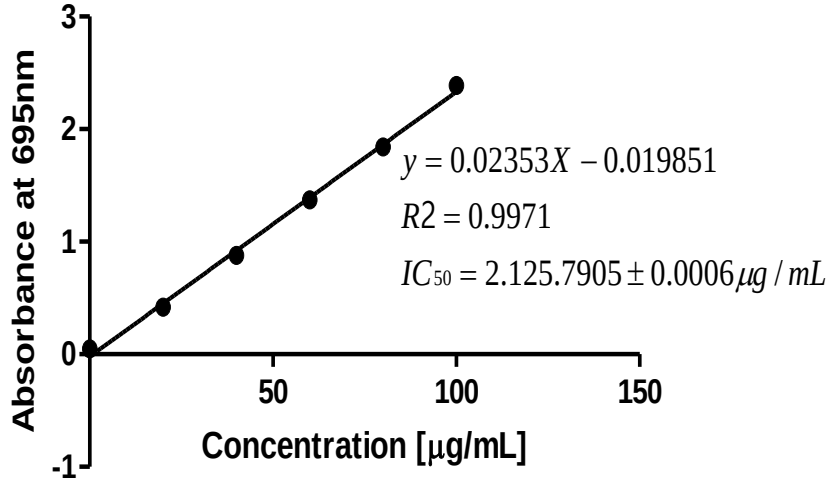

Figure 2. Antioxidant activity of Ascorbic acid.

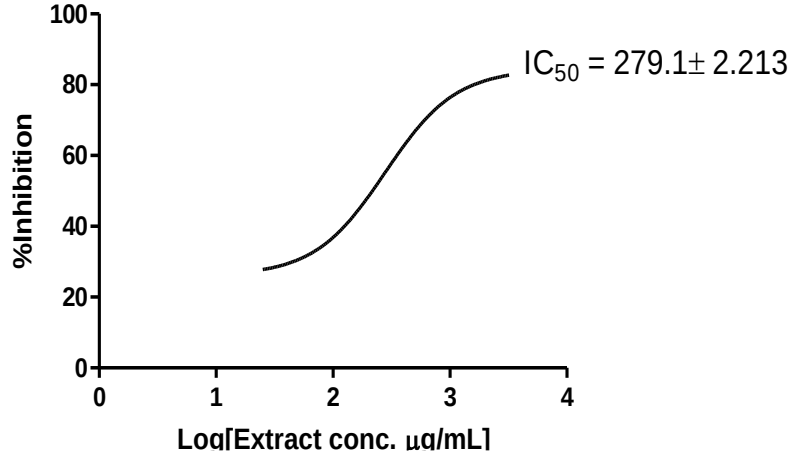

Figure 3. Anti-inflammatory activity of Tanzanian *H. abyssinica.*

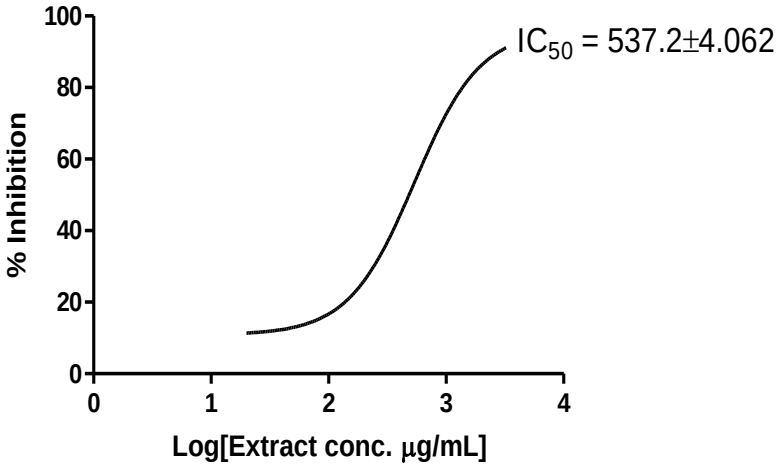

Figure 4. Anti-inflammatory activity of diclofenac sodium.

This finding is further supported by the presence of polyphenols, which are known for their numerous biological properties, among the identified compounds in the rhizomes of *H. abyssinica* (Bhattacharya, 2011).

Conclusion

The present preliminary study demonstrates that the ethanolic extract of *H. abyssinica* exhibits significant *in vitro* anti-inflammatory effects against protein denaturation, indicating its potential as an effective anti-inflammatory agent. Further research is recommended to elucidate the underlying mechanisms and compare its activity with existing anti-inflammatory agents. Additionally, researchers are encouraged to explore other medicinal plants used by traditional practitioners for treating various ailments, with the goal of discovering new therapeutic agents and validating traditional remedies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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