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Research Article

IN VITRO ANTIOXIDANT AND ANTICANCER EFFICACIES OF ETHANOLIC FRUIT EXTRACT OF *ZIZIPHUS JUJUBE* MILL

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ABSTRACT

Objective: Antioxidant and anticancer studies of the ethanolic fruit extract of *Ziziphus jujuba* were aimed at detecting the phytochemicals and ascertain the antioxidant and anticancer activities using *in vitro* experimental models.

Materials and Methods: In this study, physiochemical parameters were carried out, and the content of phytoorganic constituents such as total carbohydrates, fats, proteins, crude fibers, and flavonoids was determined. Liquid chromatography–mass spectrometry (LC–MS) has been used for detecting the phytochemicals, and 10 different concentrations have been screened for their antioxidant and anticancer activities.

Results: Ethanolic fruit extract of *Z. jujuba* revealed the presence of caffeic acid hexoside dimer, chlorogenic acid, and triterpenoid derivative by LC–MS analysis. The extract exhibited the maximum antioxidant activity of 25.32% at 10 mg/ml in 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assays, which are comparable to that of standard butylated hydroxytoluene. High concentration (10 mg/ml) of the extract revealed anticancer efficacy up to 40.36% by MTT assay, which is equivalent to the action of 5-flurouracil.

Conclusion: The ethanolic fruit extract of *Z. jujuba* might possess antioxidant and anticancer activities owing to the occurrence of bioactive compounds.

Keywords: Anticancer activity, Antioxidant activity, *Ziziphus jujube.*

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INTRODUCTION

Every year fresh cancer cases and cancer-related demises in India are being increased. Developed countries with the highest cancer rates were in the Netherlands, Australia, the U. S. A, Germany, Italy, Canada, and France. Northern Africa has the lowest cancer rate [1]. Reactive oxygen species (ROS) and free radicals can be formed in enzymatic and non-enzymatic reactions and can play a major role in the development of tumors. A shift in the balance between pro-oxidant and anti-oxidant cause damage and alteration in many intracellular tissues and molecules especially DNA, RNA, proteins and lipids lead to oxidative stress [2]. From biomedical point of view, oxidative stress is related to human diseases such as Parkinson's, Alzheimer's, and amyotrophic lateral sclerosis (neurodegenerative disease), rheumatoid arthritis (Inflammatory disease), muscular dystrophy (cardiovascular disease), and metabolic disorders as diabetes, aging, cancer, and hypo- and hyper-functions of immune system. ROS and free radicals are derived neither from some external sources such as cigarette smoking, industrial chemicals, and exposure to UV and X-rays nor from essential normal metabolic reactions in mitochondria and peroxisomes; metabolic enzymes, xanthine oxidase and the Nox family, nicotinamide adenine dinucleotide oxidase; detoxifying enzymes; metabolic process of inflammation; and defense process of phagocytosis [3, 4].

It is the crucial time to search for novel new compounds that provide specific suitable antitumorigenic effects which can be developed as anticancer agents because a growing percentage of cancer cases is acquiring resistance to current chemotherapeutic agents. Plants are natural antioxidant and anticancer agents, unlike modern allopathic drugs. When compared to synthetic ones, natural remedies have fewer side effects and toxicity. Therefore, there is a need to develop alternative antioxidant and anticancer drugs from commonly available plant source for the treatment of oxidative stress and related other diseases. *Ziziphus* (Ber) has been accepted as useful edible fruit in Ramayana [5]. To conduct a literature review on plants used in Ayurveda and Siddha systems of medicine from the phytopharmacological point of view and to select plant *Z. jujuba* with high pharmacological potentials.

Literature studies shows that the total lipid content of *Z. jujuba* (*Rhamnaceae*) fruits are very low and has rich in phenolic compounds such as catechin and rutin [6]. It has also good amount of Vitamins A, B complexes, C, E, minerals calcium, phosphorus [7, 8] and phytochemicals such as chlorogenic acid, caffeic acid and epicatechin [9, 10]. Seeds are reported to hypnotic-sedative and anxiolytic activity [11], and permeability enhancement activity [12]. Fruits are reported to have anticancer [13] and antioxidant [14] activities; leaf has immunostimulant, cardiovascular [15], anti-inflammatory, antiulcer [16], antiallergic, antidiarrheal, hypoglycemic [17], and anti-obese [15] activities. Barks are reported to wound healing [18], antifertility [19], and antimicrobial [20, 21] activities. Roots and stems are an antimicrobial activity [20, 21].

MATERIALS AND METHODS

Sample collection

Z. jujuba fruits were collected from the local market and authenticated using the Flora of the Carnatic Tamil Nadu by the Botanist Professor P. Brindha, Associate Dean and Coordinator, CARISM, SASTRA Deemed University, Tirumalaisamudram. Plant material was washed under running tap water and then sample dried in shaded condition for 2 weeks and milled to a fine powder using domestic mixer grinder.

Preparation of ethanol extract

The fine powder of the sample was taken with 70% ethanol and kept at room temperature for 2 days. The content was filtered using Whatman No. 1 filter paper, and then, the filtrate was kept for 3 days on a water bath, and the dry extract was dissolved in 70% ethanol at 10 mg/ml ratio and used for other experiments.

Physicochemical analysis [22]

Estimation of ash content

Weighed 2–3 g of the air-dried material and ignited in a muffle furnace at a temperature not exceeding 450°C cooled in a desiccator and weighed.

$$\text{Loss on drying (\%w/w)} = \frac{\text{Loss in weight (g)}}{\text{Mass of the sample (g)}} \times 100$$

Estimation of Moisture content

Weighed a Glass Stoppered Petri dish and transferred the specific quantity of sample in the dish, covered, and accurately weighed. The loaded dish is placed in the drying chamber. After drying is completed, cooled, and weighed.

$$\text{Loss on drying (\%w/w)} = \frac{\text{Loss in weight (g)}}{\text{Mass of the sample (g)}} \times 100$$

Phytochemical screening

Preliminary phytochemical identification was carried out on the presence of various secondary metabolites using standard procedures for preliminary alkaloids, saponins, glycosides, sterols, flavonoids, tannins, volatile oils, proteins, and mucilage [23].

LC–MS/MS

For the qualitative purpose, the rough sample was liquefied in ethanol (1 mg/mL) and filtered using 0.45 μm syringe filter. This solution (500 μL) was analyzed by liquid chromatography/electrospray ionization/mass spectrometry/tandem mass spectrometry (LC/ESI/MS/MS) using ultra-high-performance liquid chromatography (UHPLC) + focused reversed-phase liquid chromatography coupled to mass spectrometer (micrOTOF-Q II, Bruker, Germany). LC separations were carried out on a reverse-phase column (120 Å, 2.1 × 150 mm Acclaim 120, UHPLC + Ultimate 3000 series, Dionex). The UV detector was set at 260 nm. Mass spectrometer with ESI ionization at negative mode equipped with HyStar 3.2 software was augmented to identify the mass and fragmentation form of eluted compounds. TIC spectra were elaborated using the HyStar software. MS/MS experiments were carried out by auto-scanning mode.

Quantitative estimation of total carbohydrates [24], proteins [25], fats [26], crude fiber [27], and flavonoid content [28] was determined.

Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to analyze the antioxidant property [29].

Anticancer activity

The anticancer activity of solvent compounds was evaluated in A-549 (human lung cancer cell line) using MTT assay [30].

$$\%\text{Growth Inhibition} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of the control group}} \times 100$$

RESULTS

Physicochemical analysis

The percentage of phytoconstituents in crude drugs is mentioned on air-dried basis. In Table 1, the data obtained on the physicochemical parameters as a loss on drying and ash value were recorded.

Data obtained from the results of preliminary phytochemical screening (Table 2) revealed the presence of certain important phytochemical constituents such as alkaloids, glycosides, flavonoids, tannins, glycosides, and proteins in the plant selected.

Quantitative estimation of organic constituents in the selected plant drug revealed the presence of high and moderate levels of total carbohydrates, proteins, fats, crude fiber, and flavonoids (Table 3).

Liquid chromatogram and mass spectral studies were carried out to detect the major phytoconstituents present in the extract. The chemical constituents along with their chemical name, molecular weight, retention time (RT), and structures are tabulated in Table 4. The chromatogram and the double mass spectrum of the test drug are shown in Fig. 1.

LC–MS/MS analysis ethanolic extract of *Z. jujube* fruit

Antioxidant activity

The ethanolic fruit extract of *Z. jujube* revealed the maximum effect of 25.32% at 10 mg/ml in DPPH free radical scavenging assay, BHA, a standard antioxidant exposed the extreme inhibition 94.67% at the concentration of 10 mg/ml (Table 5).

Anticancer activity

At different concentrations (10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, and 0.02 mg/ml) of extract (100 μl), percentage of inhibition increased from 8% to 40%. 5-Flurouracil, a standard exposed the extreme inhibition 85.53% at the concentration of 10 mg/ml (Table 6).

DISCUSSION

Physicochemical analysis

Ash is the inorganic residue, which represents the total minerals content of the drugs. Loss on drying is a measure of the amount of moisture and volatile matter in a sample under specified conditions.

Preliminary phytochemical investigations of the material indicated the existence of major secondary metabolites which possess the potent therapeutic activity. The therapeutic effect or the medicinal value of any particular plant drug depends on the nature of secondary metabolites present in it. Data obtained from the results of preliminary phytochemical screening revealed the presence of certain important phytochemical ingredients such as alkaloids, glycosides, flavonoids, tannins, glycosides, and proteins in the plant selected and the extract proved to be rich in chemical contents.

Data obtained on the selected plant source revealed the presence of good amount of carbohydrates, proteins, fats, fiber, and flavonoids that play major roles in metabolism and are required in large amount.

Table 1: Physicochemical constants of the ethanolic fruit extract of *Ziziphus jujuba*

Serial number	Physicochemical parameters	Value % W/W
1	Loss on drying	7.4349
2	Total ash	38.3557

Table 2: Preliminary phytochemical screening of the ethanolic fruit extract of *Ziziphus jujuba*

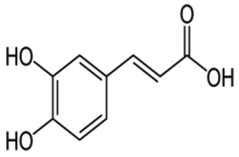
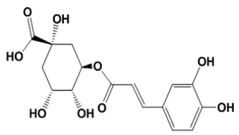
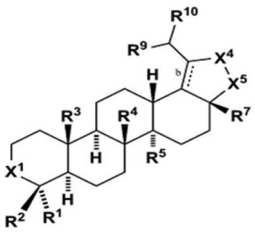
Serial number	Tests for	E
1	Alkaloids	+
2	Saponins	–
3	Glycosides	+
4	Sterols	–
5	Flavonoids	+
6	Tannins	+
7	Cardiac glycosides	+
8	Volatile oil	–
9	Cyanogenetic glycosides	+
10	Proteins	+
11	Mucilage	–

+: Presence, –: Absence, E: Ethanol extract

Table 3: Major organic constituents of ethanolic fruit extract of *Ziziphus jujuba*

Serial number	Organic constituents	Quantity (mg/g)
1	Total carbohydrates	12.382
2	Total proteins	10.6394
3	Total fats	0.394
4	Total crude fibers	2.9173
5	Total flavonoids	0.6232

Table 4: Compounds identified by liquid chromatography with tandem mass spectrometry in the ethanolic extract of *Ziziphus jujuba* fruit

Serial number	RT (min)	Compound	[M−H]	[M+H]	Structure	MS/MS (production)	Reference
1	3.0–3.2	Caffeic acid hexoside dimer	683.2	-		341.1	VitorSpinola *et al.*
2	10.8	Chlorogenic acid	353.2	-	 Chlorogenic Acid	173, 179, 271, 284, 307,353	Massbank database (KO000466)
3	5.4–5.6	Triterpenoid derivative	-	663.4		551.3, 495.3, 439.2	Ibrahim Abu Reidah *et al.*

RT: Retention time, MS/MS: Tandem mass spectrometry

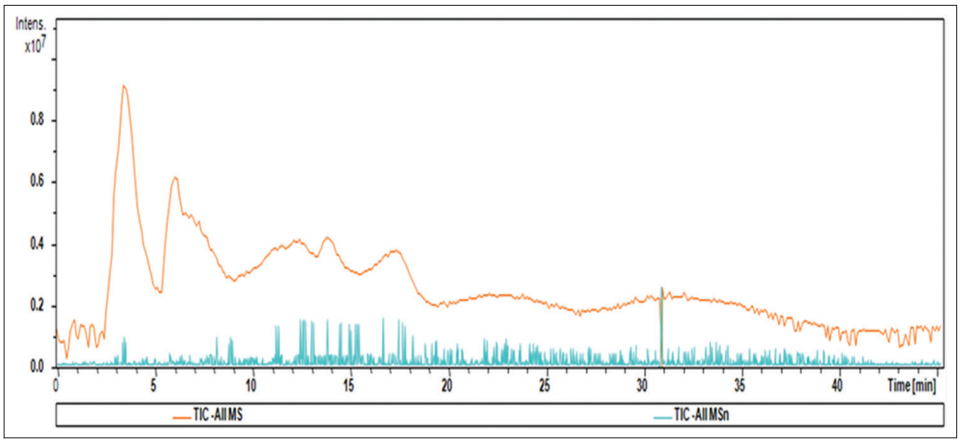


Fig. 1: Total ion chromatogram (negative mode) obtained from Liquid chromatography–mass spectrometry (LC–MS)

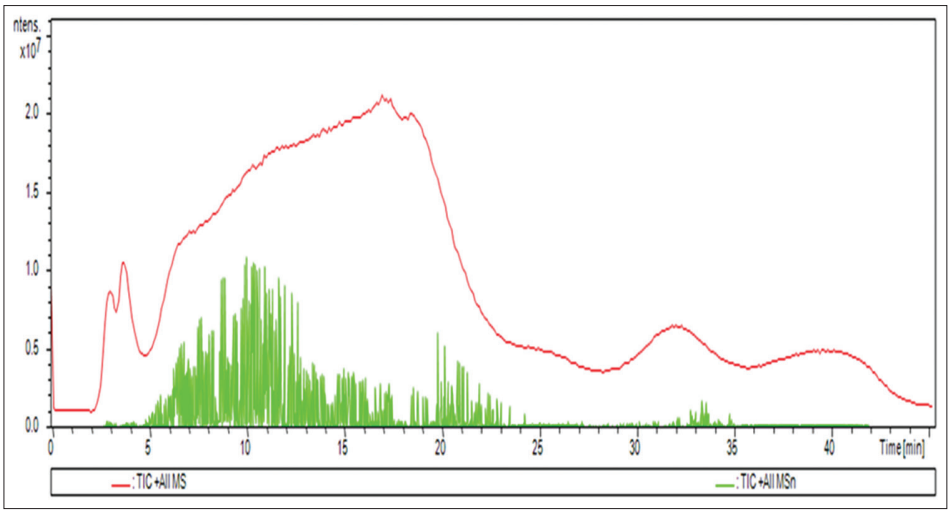


Fig. 2: Caffeic acid (negative mode) obtained from LC–MS

Carbohydrates are hydrolyzed using dilute hydrochloric acid to produce simple sugars. Simple sugar is dehydrated to hydroxymethylfurfural in hot acidic medium. These compounds produce a green-colored product with Anthrone reagents [31]. Deficiencies or excess of nutrients, especially proteins, carbohydrates, and vitamins, leads to various complications and metabolic disorders in human beings. The proteins will act as a building block of receptors, hormones, enzymes, tissues, etc. Crude fiber will serve as hypolipidemic and hypoglycemic agents. It also reduces the danger of hypertension, coronary heart disease, and colon and breast cancer. The suggested dietary levels of fibers essential for children, adults, and pregnant and lactating mothers are 19–25 g/d, 21–38 g/d, and 28–29 g/d, correspondingly [32]. Flavonoids present in the test drugs will enhance the effects of Vitamin C and functions as antioxidants [33].

LS–MS is a key enabling technology for the detection and characterization of organic molecules. In the present study, LC–MS analysis of *Z. jujube* fruit was carried out to detect the possible chemical components present in them Fig. 1. In the ethanolic extract, chemical components such as caffeic acid hexoside dimer them Fig. 2, chlorogenic acid them Fig. 3, and triterpenoid derivative them Fig. 4 were identified.

Antioxidant activity

Antioxidants play a central role in defusing free radical species which are formed from various biochemical reactions in normal system. These free radicals are the main culprits in lipid peroxidation. The production of ROS and free radicals occurs in various diseases, which directly/indirectly activate phagocytic cells [34]. The above mentioned process initiates lipid peroxidation and leads to membrane destruction. The synthetic medicines are known to produce severe side effects in the body such as heart attacks and stroke. Hence, robust limitations have been positioned on their application, and there is a drift to substitute them with phytomedicine. Phytodrug having antioxidant property is non-toxic or may have minimum side effects than synthetic compounds. In this concern, we attempt to search out an herbal drug to substitute synthetic ones. Antioxidant in plant-drug reacts with DPPH (purple color), a stable free radical and reduce it to DPPH:H form of yellowish-colored diphenyl picrylhydrazine derivative [30]. The degree of discoloration shows the scavenging effect of the antioxidant compound [35].

The ethanolic fruit extract of *Z. jujube* exhibited the maximum activity of 25.32% at 10 mg/ml in DPPH free radical scavenging assay, BHA, a standard antioxidant exposed the extreme inhibition 94.67% at the concentration of 10 mg/ml (Table 5). The activity was dose dependent, which is amplified with a rise in the concentration of the extract. Such radical scavengers may well-safeguard tissues from ROS and thus thwart oxidative impairment linked illnesses.

Anticancer activity

Cancers are one of the most dangerous roots of death among humans throughout the globe and their relative importance, and their awareness is also gradually increasing. The anticancer activity was evaluated in human lung cancer cell line (A-549) using 3-(4,5-dimethylthiazol-2-yl)-

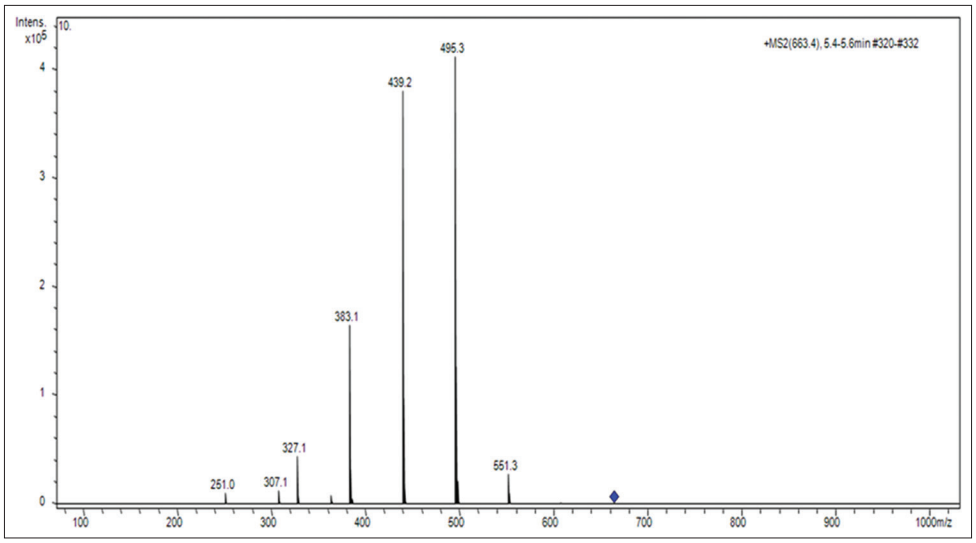


Fig. 3: Hexoside dimer chlorogenic acid (negative mode) obtained from LC–MS

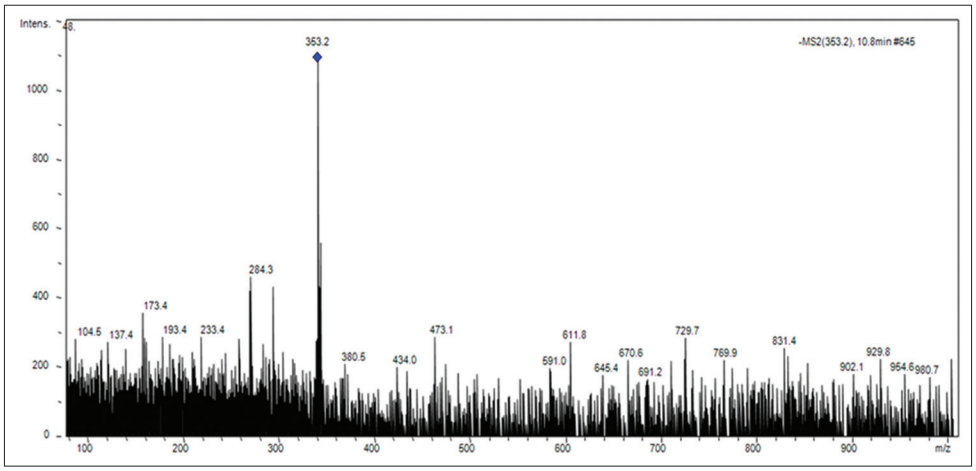


Fig. 4: Triterpenoid derivative (positive mode) obtained from LC–MS

Table 5: 1, 1diphenyl-2-picrylhydrazyl radical scavenging activity of the ethanolic extract of *Ziziphus jujuba* fruit

Serial number	Concentration of the sample (mg/ml)	Antioxidant activity (%)	
		Extract	Standard (BHA)
1	10.00	25.32±1.77	94.67±2.14
2	5.00	21.64±0.95	93.72±1.05
3	2.50	19.58±0.76	91.39±2.46
4	1.25	17.42±0.58	93.33±1.23
5	0.63	13.39±1.21	84.00±1.12
6	0.31	10.02±0.33	64.17±1.27
7	0.16	9.27±0.84	45.33±1.41
8	0.08	8.69±0.57	30.67±1.52
9	0.04	7.21±0.65	21.33±0.59
10	0.02	5.15±0.46	17.33±0.62

Values are mean±SD (*n*=3). SD: Standard deviation, BHA: Butylated hydroxyanisole

Table 6: Anticancer activity of *Ziziphus jujuba* fruit against lung cancer cell line

Serial number	Concentration of the sample (mg/ml)	Anticancer activity (%)	
		Extract	Standard (5-fluorouracil)
1	10.00	40.36±0.85	85.53±1.83
2	5.00	39.18±0.42	82.40±1.35
3	2.50	38.63±0.60	78.45±1.04
4	1.25	35.47±1.03	72.28±1.02
5	0.63	31.56±0.55	65.60±1.06
6	0.31	27.75±0.23	59.72±1.18
7	0.16	22.86±0.61	50.23±1.22
8	0.08	18.29±0.56	43.30±1.29
9	0.04	13.78±0.74	35.82±0.14
10	0.02	8.25±0.09	28.98±0.31

Values are mean±SD (*n*=3). SD: Standard deviation

2,5-diphenyltetrazolium bromide (MTT) assay. Research can focus on ascertaining drugs that influence cell growth and metabolism or induce cell death. Stem cell-based treatments can rely on understanding cells differentiation and cell interaction. In MTT assay, the dead cells or their yields do not condense tetrazolium. This assay based on the number of cells existent and the mitochondrial action/cell. The principle involved in MTT assay is the cleavage of a tetrazolium salt into a blue-colored formazan by Succinate dehydrogenase as one of the mitochondrial enzymes. The quantity of cells was found to depend on the level of formazan making by the cells used. Results of MTT assay are tabulated in Table 6. At different concentrations (10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, and 0.02 mg/ml) of extract (100 µl), percentage of inhibition increased from 8% to 40%. The highest percentage of inhibition (40.36%) was obtained at the concentration of 10 µg/ml. The results suggested that the plant drug inhibited the proliferation of human A549 (lung carcinoma) cells.

CONCLUSION

The present studies can be decided from the outcomes, the ethanolic fruit extract of *Z. jujuba* might possess antioxidant and anticancer activities due to the presence of phytoconstituents. Further studies are needed for the isolation and identification of active compounds and explore the mechanism of action.

AUTHORS' CONTRIBUTIONS

Both Akila B and Manikandaselvi S have carried out experiments, analyzed results, and typing the manuscript.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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