Cultivation, Nutritional, Micro-nutritional and Ursolic acid Quantification (HPTLC) in Organic and Conventional *Ocimum sanctum*

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Abstract:

The demand for medicinal plants is growing at the rate of about 15 % per annum. Currently a major part of it is contributed by wild sources, however with biodiversity norms 2002 in action; cultivation of medicinal plants has become indispensible to satisfy the requirement of industry. Until now no scientific studies are conducted to ascertain nutrition, phyto-constituents and safety measures of organically grown medicinal plants. In this research, *In vivo* studies were conducted to assess the morpho-physiological traits of known Holy medicinal plant *Ocimum sanctum* using organic and non-organic farming techniques. The experiment was performed in Randomized Block Design with twelve replications using (organic & non-organic) fertilizer and pesticides. The aerial parts were harvested; lyophilized and dried coarse powder was screened for nutritional, anti-nutritional, physico-chemical and mineral (by ICP-AES). The water extracts were quantified for the presence of Ursolic acid by using HPTLC. The mean performances of all the traits were higher in organic Tulsi (OOS) except the weight of plant material. Proximate analysis revealed the high caloric value (235.2 Kcal/100g DW) along with protein, lipid and crude fibers in OOS. Similarly, high mineral contents (K, Ca, Mg, Fe, Na, Zn, Cu and Mn 181.94, 45.64, 23.83, 39.35, 159.4, 75.82, 5.3 and 4.61 mg/100 g DW respectively) were found in OOS. Whereas non-organic Tulsi (NOS) contained high content of total ash, extractives, oxalate, phytate and heavy metals (Pd – 1.82 mg/100g DW). The higher amount of Ursolic acid content was found in OOS 0.17 % w/w by HPTLC method. Evidence proved the perception that organically grown foods or medicinal herbs are 'better for you' in terms of nutrition, phyto-constituents, better quality and safety measures.

Keywords:
Cultivation, HPTLC, Micro-nutritional, Nutritional, Ursolic acid.

Introduction:

Biological Diversity Act (2002) and Rule (2004) enforced the noble thought of protecting our biodiversity especially crude drugs from plant origin [1]. Therefore it is the need of current era, to produce these crude drugs by cultivation/farming in our fields. A series of food scares and the controversy surrounding genetically modified crops have prompted heated debate about the safety and integrity of our food and herbal medicines. Against this background, demand for organically grown food has been growing rapidly [2]. Increasing awareness about health hazards associated with agrochemicals and

consumer's preference to safe and hazard-free food are the major factors that leads to the growing interest in alternate forms of agriculture in the world. Organic cultivation is one among the broad spectrum of productive methods that are supportive to the environment. The demand for organic food is steadily increasing both in the developed as well as developing countries with an annual average growth rate of 20–25 % [3]. Therefore, the present study was designed to scientifically validate the perception that organically cultivated food or medicine are safe and better in terms of growth pattern, nutrients, micro-nutrients and phytocontituents by taking *Ocimum sanctum* as an experimental model.

Material and methods:

The present investigation was carried out in the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, situated in the centre of the Indian peninsula 79° 7´ East longitude and 21° 7´North latitude and is at a mean altitude of 310.5 meters above sea level. The average annual rainfall of the area is 1205 mm. The average maximum temperature of the city is 33.53°C and the average minimum temperature is 20.37°C and the relative humidity ranges from 20 to 70 % [4]. Seeds were procured from Organic India Pvt. Ltd. Lucknow, India.

1. Experimental land

The land for the organic treatment was selected and converted (period 2.5 years before 1st harvest) as per the recommendations of National Centre for Organic Farming, India [5] and the parallel area with marked buffer zone was selected for conventional treatment. The soil of experimental site was comprised of black clay soil with a pH of 6.8, rich in humus, potash and trace elements (Cu, Fe, Mn and Zn,) [analyzed by 'Soil Survey and Soil Analysis Department' Government of India, Nagpur, MS, India].

2. Manures

Organic manure was prepared using a mixture of protein rich materials including animal manures, fresh green grass, leaves and shoots of leguminous plants or trees (*Cassia* species) and carbon rich materials including wood chips, dry leaves and grasses in a proportion of 60 % protein and 40 % carbonaceous. The heap was made by a layering method, of about 2 m wide at the base, 0.5 m high and 3 m long, comprised of first stiff / hard layer of woody stems as a base, later the carbonaceous layer about 10 cm deep, alternating with the loose proteinaceous layer which was about 15 cm deep. The animal dung was made into slurry using cow urine and watered onto the carbon layer. Very little amount of hydrated lime and rock powder were sprinkled over the pile. Heap was turned regularly after about 6 weeks and brown, crumbly humus was ready in 3 months [6]. Non-organic fertilizer viz.

urea granules (N), super phosphate (P_2O_5) and potash (K_2O) per hectare were applied according to the treatment schedule.

3. Pesticides

3.1 Organic insecticide and fungicide

Fully dried *Azadirachta indica* L. (neem) seeds of about 500 g were pulverized and macerated for 24 hr in 10 litres of Milli Q water (Bio AGE Direct Ultra, Punjab, India). Filtrate was 362 utilized as potent insecticide. Fresh *Allium sativum* L. (garlic) paste was prepared and about 250 g paste was fermented for about 15 days in 05 litres of Milli Q (MQ) water. The fermented filtrate was diluted again with 05 litres of MQ water and sprinkled over organic crop as fungicide [7].

3.2 Non-organic insecticide and fungicide

Monocrotophos 36 % (Monocil, Insecticide India Ltd. India) as an insecticide and Zineb 75 % W.P. (Indofil Z-78, Indofil Industries Ltd, Mumbai, India) as an fungicide were purchased from local market and utilised in a ratio of 3:2 in MQ water respectively as per product manual [8].

4. Cultivation and harvesting

All the seeds were sown in their favourable season with the implementation of good agricultural practices by adopting the randomized block design in twelve replicates of each treatment in the year 2013. Two different treatments were utilized, organic and non-organic (in terms of fertilizer and insecticide/fungicide). About 5 m^3 per acre organic (biodynamic manure) was utilised for the organic crop. Non-organic manure (Urea, Super phosphate and potash) was utilised in a ratio of 120:105:105 respectively [8]. Half of the N and entire P were applied at basal stare and the remaining of N was applied in two splits at vegetative and flowering stage. About 10 litres per acre of each organic and non-organic pesticide were sprayed. Whole plants of organic and non-organic *Ocimum sanctum* (OOS & NOS) were harvested at the stage of maturity (flowering condition) on the 90th day after planting the seedlings [9]. The two morpho-physiological traits (plant height (inches) and secondary branches) were observed with the interval of 10 days till harvesting (pre-harvesting) and five traits (plant height (inches), secondary branches, weight of whole plant, roots length (inches) and secondary roots) were examined after harvesting (post-harvest: p.h.) using statistical package PAST (Version 2.03). The plant was botanically authenticated from the Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur. Voucher specimen (9783) has been deposited for future reference. The aerial parts of plant were cut into small pieces and shade dried. The dried material was pulverized and coarse powder was stored in air tight containers for further use.

5. Proximate analysis

Moisture, Lipid, protein, fiber and ash contents were determined as per AOAC methods [10].

5.1 Carbohydrate and caloric value

The total carbohydrate content (g/100 g) in the samples was calculated by difference method. The caloric value was calculated by sum of the percentages of proteins and carbohydrates multiplied by a factor of 4 (kcal/100 g) and total lipids multiplied by a factor of 9 (kcal/100 g) [11].

6. Anti-nutritional analysis

6.1 Phytate content

Phytate of each sample was determined according to the method described by Maga [12].

6.2 Oxalate content

The titration method was used to determine the oxalate content according to the methods of Day and Underwood [13].

7. Mineral analysis

The total 12 minerals i.e. Na, K, Ca, Mg, Fe, Zn, Cu, Mn, Pb, Cd, As and Hg were determined with an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP- AES; ARCOS, Spectro, Germany) and sample were prepared by acid digestion method [14].

8. Extraction and phytochemical screening

The hot water extracts (decoctions) were prepared by heating the well dried crude powder of aerial parts of OOS & NOS (100 gm each) with 1000 ml of milli Q water (BioAGE Direct Ultra, Punjab, India) at 80oC for about 3h with constant stirring. The aqueous portion was collected, cooled, filtered, concentrated in vacuo (25°C) (SONAR Buchitype) and then lyophilized to dry (LYODEL, model-DPRG-1GH). Extracts were screened for the presence of polyphenols, saponins, phytosterols, alkaloids, flavonoids, proteins and carbohydrates by using different phytochemical testings [15].

9. HPTLC quantification of Ursolic acid

9.1 Equipment and chromatographic condition:

A CAMAG HPTLC system equipped with a sample applicator Linomat IV using 100 ml syringe and connected to a nitrogen tank; twin trough plate development chamber; CAMAG TLC scanner-3 with win CATS software. Each HPTLC plate precoated, silica gel G 60 F254 size 10 X 10 cm accommodated eight tracks of standards and samples, applied according to following settings: bandwidth 4 mm; distance between bands 5 mm; The plates were developed to 8 cm in a twin trough glass chamber, saturation time 30 min, scanning mode Absorbance/Reflectance; temperature 20±5 °C and separation technique ascending.

9.2 Chemicals:

Standard ursolic acid (Yucca Enterprises, Mumbai), precoated silica gel G 60 F254 TLC aluminium plates (20 X 20 cm, 0.2 mm thick) (Merck Ltd. Germany) and AR grade chemicals were used. The marketed samples were purchased from local market of Nagpur, Maharashtra.

9.3 Preparation of standard and sample

9.4 Ursolic acid stock solution of 2 mg/5 ml was prepared in water and volume was made to 10 ml and different amounts 1 to 7 µL were applied. The OOS and NOS extracts 10 mg/ml was prepared and 1 µL of each was on TLC plate using a CamagLinomat V sample applicator [16].

9.5 Calibration curve

Calibration curve was constructed according to requirement of ICH guidelines. Each concentration was applied to a plate (20 X 10 cm) in triplicates of 6 mm band length with a distance of 5 mm between each two bands. The distance from the plate side edge was 10 mm and from the bottom of the plate was also 10 mm. The application rate was 25 µL/s, standard zones were quantified by linear densitometric scanning using Camag TLC scanner. Deuterium lamp was utilized as a source of radiation. Evaluation was done using linear regression analysis via peak areas and calibration curve was prepared by plotting peak area vs. concentration applied [16].

10. Statistical analysis

Statistical analysis was conducted using statistical package PAST (Version 2.03) for morpho-physiological traits and GraphPad Prism version 5.02 (for Windows) for proximate, anti-nutritional and mineral analysis. All the determinations were carried out in triplicate and data were expressed as mean ± (n = 3) standard deviation.

Result and discussion:

1. Morpho-physiological characteristics and their mean performance

The mean performance and range (pre-harvest and post-harvest – p.h.) for 5 different morpho-physiological traits of 12 tulsi replicates (organically and non-organically grown) are represented in Table 1 and 2. All the traits including plant height (inches) [Fig. 1], number of sec. branches, roots length (inches), number of secondary roots and weights (g) were found to acquire higher mean values of 35.70, 26.16, 17.83, 10.83 and 244.63 (p.h. 105th day) respectively in OOS. Among 12 Tulsi replicates under organic and non-organic conditions, the OOS showed two days early inflorescence and exhibited higher mean performance in all the morpho-physiological traits except only less average yield of plant (244.63 g/12 replicates), while NOS given higher average yield of plants (279.61 g/12 replicates). OOS showed early germination, secondary branches and sustain life which may be attributed to the presence of plenty of 'beneficial soil microbes' in organic manure which helps in 'soil regeneration' & 'fertility improvement' and protect them from degradation while also promoting growth in plants [17].

2. Proximate analysis

The dried aerial parts of OOS contained higher content of protein, lipid, crude fibers and caloric value (235.20 kcal/100 g DW) [Table 3], indicating the

enrichment of energy source in the organic crop. The higher carbohydrate content in OOS (44.10 % DW) was the highest calorie contributor as the total lipid and protein contents did not considerably affect the determination of energy produced [Table 3].NOS showed the higher amount of total ash (17.9 % DW) and water soluble extractives (22.8 % DW) which may be a primary indicator of high heavy metal content and other inorganic salts [Table 3].

3. Anti-nutritional analysis

Similarly the anti-nutritional factors viz. oxalate (11.2 g/100g) and phytate (0.19 g/100g) were found to be higher in NOS [Table 3]. Phytic acid strongly binds to metallic cations of Ca, Fe, K, Mg, Mn and Zn making them insoluble and thus unavailable as nutritional factors [18].

It is reported that organic acids and amino acids (such as citric acid, tartaric acid, oxalic acid, succinic acid, aspartic acid and glutamic acid) excreted by the roots of plant formed soluble complexes with heavy metals and increased the mobility of heavy metals in soil [19].

4. Mineral analysis

Organic plants were found to contain higher contents of all the 8 minerals examined such as K, Ca, Mg, Fe, Na, Zn, Cu and Mn [Table 4]. The high content of potassium compared to sodium leads to a very low Na/K ratio, which is favorable from nutritional point of view, as diets with low Na/K ratio are associated with lower incidence of hypertension [20]. Followed by calcium, which was found to be the second most abundant mineral element in the OOS, therefore it can be considered as an appropriate dietary source of calcium to maintain the biological role of nerve transmission, muscle contraction, glandular secretion as well as mediating vascular contraction and vasodilation [21].

A higher bioavailability of the dietary iron can be achieved by increasing the content of food components enhancing iron absorption (ascorbic acid) or by decreasing the content of inhibitors (e.g., phytates, oxalates and tannins) [22]. The optimum amount of iron observed in tulsi indicated that the plant could be a good source of dietary iron to overcome nutritional deficiency of iron, if supplemented in the diet. Excessive ratio of zinc to copper (>16) from dietary sources causes imbalance in their bioavailability and has been linked to increased risk of cardiovascular disorders [23]. OOS with its zinc/copper ratio of four (6.38) compared to NOS (7.45), represents a potential food source to counter copper-zinc imbalances.

Dried aerial parts were also examined for the detection of toxic heavy metals such as Pb, Cd, As and Hg. Heavy metals such as Cd and Pb are non-essential elements for plants. If higher amounts are accumulated in the plants, heavy metals will adversely affect the absorption and transport of essential

elements, disturb the metabolism and have an impact on growth and reproduction [24]. OOS showed the very negligible amount of Pb (0.2 mg/100g DW) while NOS contained Pb (1.8 mg/100g DW respectively) that signifies the lower concentration of minerals in NOS [Table 4].

5. Qualitative phytochemical screening

Both the extracts were found to contain carbohydrates, proteins, fats, polyphenols, flavonoids and saponins.

6. HPTLC analysis

Both the extracts were quantified for the ursolic acid content with help of HPTLC linearity curve of standard ursolic acid using mobile phase toluene: ethyl acetate: glacial acetic acid (09:01:0.4) [Fig. 2]. The higher amount of ursolic acid content with Rf of 0.44 was found in OOS (**0.17 % w/w**) while NOS contained 0.16 % w/w of ursolic acid attributed high quality of organic Ocimum sanctum in terms of secondary metabolites [Fig. 3].

Table 1: Statistical Data of Organic *Ocimum sanctum*

Traits of OOS	Days	Range		Mean	Std error	Std Dev	Variance
		Min	Max				
Height (inch)	30	2.5	7	4.86	7.42	0.10	11.52
	40	7.5	19	11	0.11	0.16	12.22
	50	14	26	19.70	0.099	0.14	7.62
	60	17	26	22.08	0.058	8.20	4.26
	70	20	30	24.45	0.071	0.10	4.93
	80	23	32	27.08	0.083	0.11	5.53
	90	23.5	34	29	0.11	0.15	7.12
	100	27	37.5	32	0.12	0.17	7.56
	105	32	39.5	35.70	6.71	9.50	3.91
Sec branch	30	0	0	0	0	0	1.70
	40	9	16	12	0.86	1.22	21.16
	50	10	22	14.66	0.79	1.12	15.77
	60	12	28	20.5	2.29	3.24	39.39
	70	14	30	21.83	1.44	2.03	23.64
	80	15	36	23.33	1.76	2.50	26.89
	90	18	30	23.75	1.41	2	21.07
	100	18	38	27.5	1.22	1.72	16.32
	105	19	33	26.16	2.03	2.88	27.44
Weight (gm)	105	156	455	244.6	39.87	56.3	52.69
Roots length (inch)	105	14	19.5	17.83	0.49	0.69	10.13
Sec roots	105	8	15	10.83	0.51	0.73	16.97

Table 2: Statistical Data of Non-organic *Ocimum sanctum*

Traits of NOS	Days	Range		Mean	Std error	Std Dev	Variance
		Min	Max				
Height (inch)	30	3	5.5	4.20	0.33	0.47	25.48
	40	7	13	9.79	0.80	1.14	26.91
	50	16	25	20.25	0.89	1.27	15.60
	60	18	24	21.54	0.54	0.77	8.70
	70	20	25	24.41	0.71	1.01	10.12
	80	23	30	27	0.87	1.23	11.21
	90	22	33.5	28.70	1.19	1.68	14.30
	100	23.5	37	31.12	1.37	1.94	15.09
	105	28	37	34.04	0.78	1.10	7.78
Sec branch	30	0	0	0	1.66	2.34	8.13
	40	10	23	16.41	0.10	0.15	9.94
	50	16	25	20.25	0.093	0.13	7.68
	60	15	30	19.83	0.25	0.35	19.28
	70	17	24	20.41	0.15	0.22	11.74
	80	12	28	22.25	0.18	0.26	13.50
	90	12	36	22.75	0.14	0.19	10.09
	100	12	36	24.33	0.12	0.18	8.81
	105	15	40	25.25	0.19	0.27	13.50
Weight (g)	105	148	750	279.61	1.04	1.48	22.85
Roots length (inch)	105	9	19.5	15.70	6.30	0.089	5.27
Sec roots	105	6	14	10.33	8.20	0.11	8.62

Table 3: Proximate, Nutritional and Anti-nutritional Analysis

S. N.	Analysis (dried aerial parts)	OOS	NOS
1	Protein (g/100 g)	9.7	9.0
2	Fat (g/100 g)	2.2	1.4
3	Carbohydrate (g/100 g)	44.1	43.8
4	Crude fiber (g/100 g)	25.2	22.3
5	Moisture (g/100 g) (fresh whole fruits)	3.3	5.4
6	Energy (K cal/ 100 g)	235.2	224
7	Water solubleextractive value (g/100 g)	13.9	22.8
8	Total ash (% w/w)	15.2	17.9
9	Oxalates	6.4	11.2
10	Phytates	0.16	0.19

Table 4: Mineral Contents

Minerals (mg/100g)	K	Ca	Mg	Fe	Na	Zn	Cu	Mn	Pb	Cd	Hg	As
OOS	181.9	45.6	23.8	39.3	159.4	65.8	10.3	4.6	0.2	0	0	0
NOS	164.2	31.5	17.3	13.1	116.9	31.3	4.2	4.1	1.8	0	0	0

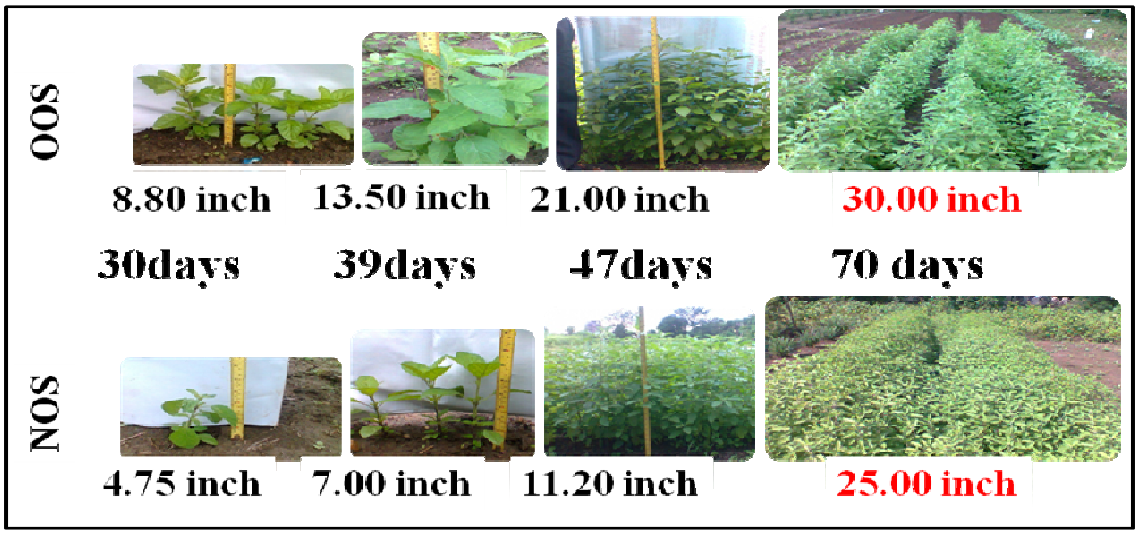

Figure 1: The performance of *Ocimum sanctum* in terms of trait (height)
Where OOS: organically cultivated *Ocimum sanctum*and NOS: non-organically cultivated *Ocimum sanctum*

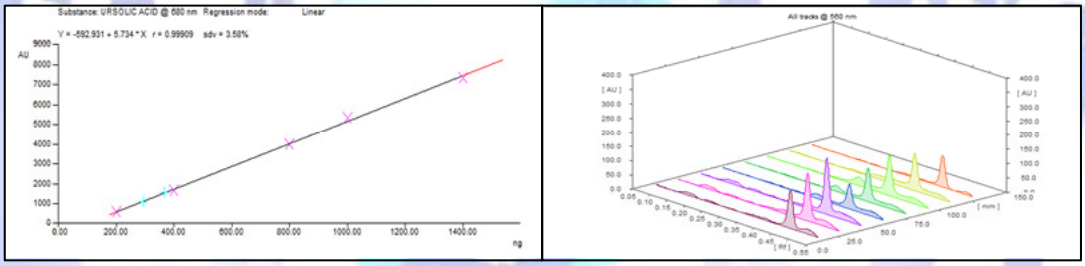

Figure 2: Linearity curve of ursolic acid and 3D Spectra

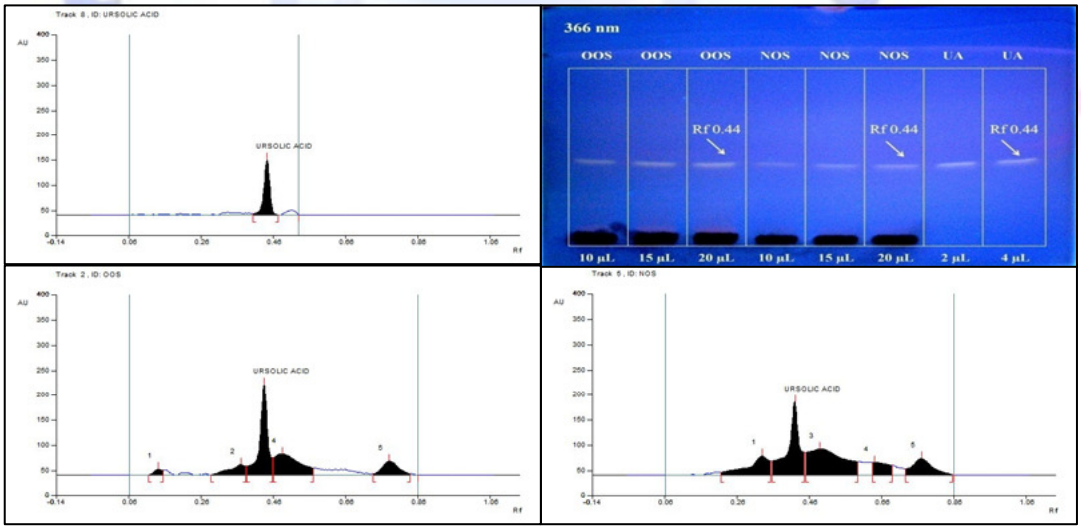

Figure 3: HPTLC Chromatograms of Ursolic acid, OOS, NOS and Plate at 366 nm

Conclusion:

This study is an attempt to set a milestone in justifying the importance of organic over non-organic medicinal plant species through the differences in their morpho-physiological traits, nutritional, anti-nutritional, minerals and phytochemical contents. Conclusive evidences generated from this research study on *Ocimum sanctum,* proved the perception that organically grown foods or medicinal herbs are 'better for you' in terms of nourishment, sustainability, better quality standards and safety measures.

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