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Short communication



Drying and Extraction Methods Effect on Biochemical and An-tioxidant Capacity of Malaysian Wild Edible Vegetables

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Abstract

Some wild edible vegetables are rarely been studied albeit freshly consumed by local people as '*ulam*'. Due to their perishable nature, drying is applied to extend their shelf life which causes substantial changes in phytochemicals content. Hence for collection of optimum yield of phenolics, the use of solvents of different polarities is crucial. In this study, the effect of drying (freeze-drying and oven-drying) and extractants solvents (ethanol and water) methods on phenolics and flavonoids contents as well as antioxidant activity of ten immature wild edible vegetables extracts (*Acrosticum aureum*, *Erechtites hieraciifolia*, *Erechtites valerianifolia*, *Gnetum gnemon*, *Manihot esculenta*, *Oroxylum indicum*, *Phyllanthus acidus*, *Piper sarmentosum*, *Terminalia catappa* and *Ziziphus mauritiana*) collected in Terengganu, Malaysia were investigated. Extraction were carried out using ultrasonication-assisted method. The results indicated that some vegetables were good sources of antioxidants with the lowest IC_{50} value of DPPH scavenging was 6.19 µg/mL. Ethanolic freeze dried (EFD) extracts (0.5 mg/mL) showed stronger DPPH scavenging activity (Inhibition rates: 76 to 88%) compared to ethanolic oven dried (EOD) (56 to 88%) and aqueous freeze dried (AFD) (35 to 89%). Total phenolics content (TPC) and total flavonoid contents (TFC) were found to exhibit strong to moderate correlations with antiradical power. Among all samples, *T. catappa* following EFD, EOD and AFD showed the strongest antioxidant potential. The findings revealed that different drying methods and extracting solvents did influence the biochemicals isolation and antioxidant activity.

Keywords: *DPPH free radical scavenging activity; drying condition; extraction solvent; total flavonoids content; total phenolics content.*

1. Introduction

Medicinal plants have important contributions in the healthcare system of local communities. The plants become the main source of medicine for the majority of rural population [1] such as in India where traditional healers use about 2500 plant species and 100 species of plant to serve as common sources of medicine [2]. Strong antioxidant, greater level of phenolics, rich in dietary fibers, minerals, vitamin C and various phytochemicals sustained the use of medicinal plants in healthcare in preventing non-communicable disease [3, 4].

In Malaysia, traditional vegetables or '*ulam*' are freshly consumed or half-boiled at a medium temperature. They contain vitamins, fibres and minerals as well as exhibit medicinal properties against diabetes, heart disease and problems with the digestive tract [5]. For example, some common '*ulam*' species namely, *Centella asiatica* (L.) Urban (pegaga), *Anacardium occidentale* L. (gajus), *Colubrina asiatica* (peria pantai), *Pluchea indica* (beluntas) and *Premna cordifolia* (bebuas)were studied for phenolic compounds and analysed for antioxidant activities [6].

There are various methods used in plant extraction which affect the extraction yield and antioxidant activity. Drying is a process of removing the moisture content to preserve the phytochemicals/ bioactive constituents and prolong their shelf life by retardation of microbial spoilage [7, 8]. Different drying methods may affect the chemicals and biological activities of the vegetables. Generally, three drying methods are used in drying process of leafy vegetables, which are freeze dry, oven dry and air dry. Freeze drying is regarded as the better method for moisture removal since the leaf is shock freezing with liquid nitrogen upon plucking in order to stop any metabolic reaction and produce highest quality of the final product. The ice crystals formation within the plant matrix can rupture the cell structures which provide the release of cellular components [9]. Oven drying would exposed excess heat on the leaves which could on lead to degradation of certain heat-sensitive compounds and resulting in reducing the chemicals amount and reaction [10, 11]. Air dry and oven dry are commonly used in herbal drying process among domestic industry due to their rapid, simple and inexpensive methods [12]. Besides, the extractant solution used may also affect the extraction yield and antioxidant capacity [13]. Polar solvents are applicable to recover polyphenols from plant samples. For examples, flavonoids from tea isolation were better carried out using water/ethanol than water/methanol and water/acetone [14].

However, the information on the effect of drying as well as extraction solvents on the antioxidant activity of Malaysian wild edible vegetables are limited. Therefore, the aim of this study was to investigate the concentrations of polyphenols compounds and antioxidant activity of ethanol and aqueous extracts of Malaysian wild edible vegetables prior to freeze and oven drying.

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2. Materials and Methods

2.1. General

Folin-Ciocalteu's (FC) reagent, sodium carbonate anhydrous, sodium nitrite, sodium hydroxide, aluminium chloride anhydrous, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, gallic acid, and bovine serum albumine (BSA) were purchased from Sigma-Aldrich GmbH (Sternheim, Germany). D-glucose, sulfuric acid (H_2SO_4), ethanol (analytical grade), methanol (analytical grade) were obtained from Merck (Darmstadt, Germany). Bradford reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenol solution was purchased from Thermo Fisher Scientific (MA, USA). Absorbances in colorimetric tests were read using ELISA microplate reader (TECAN, US). Distilled water (dH_2O) was used in related experiments. Stock solution of all samples were prepared at concentration of 1 mg/mL and diluted to a final concentration of 100 µg/mL in all chemical content assays. The standard calibration curve were established using standards with concentration ranging from 200 to 1.56 µg/mL (2-fold dilution) versus absorbances. The mean of four replicate results was used in all analysis.

2.2. Sample collection, drying and extraction

Ten wild edible vegetables; *Acrosticum aureum*, *Erechtites hieraciifolia*, *Erechtites valerianifolia*, *Gnetum gnemon*, *Manihot esculenta*, *Oroxylum indicum*, *Phyllanthus acidus*, *Piper sarmentosum*, *Terminalia catappa* and *Ziziphus mauritiana* were collected from several localities in Terengganu and authenticated by Prof Dr Nashriyah Mat, Faculty of Bioresources and Food Industry (FBIM), Universiti Sultan Zainal Abidin, Terengganu. The vouchers specimens were deposited in FBIM Herbarium. Immature leaves collected, were ensured to be free of dirt and latex, and immediately frozen in liquid nitrogen to quench metabolic reaction.

The sample was then dried using two different drying methods; Freeze-drying (FD) and oven-drying (OD) [15]. For FD method, the leaves were ground to rough powdered form under liquid nitrogen and then stored overnight at -20 °C and lyophilized (Freeze Dryer CHRIST Alpha 1-4Ldplus, Protocols; Main drying: -20 °C at 1.0 mBar, Final drying: -56 °C at 0.0018 mBar). For OD method, the leaves were dried at 37 °C for 48 hours (h) and ground to rough powdered form. Samples were stored in vacuum desiccator prior to extraction. Sample (5 g) was macerated in ethanol or water (25 mL), heated at 70 °C for 15 minutes (min), followed by sonication (15 min) and centrifugation (4000 rpm, 15 min). The supernatant was concentrated under *vacuum* and completely dried to crude extract under nitrogen gas flow. The crude extract was stored at -20 °C before analyzed.

2.3. Total phenolics content

Total phenolics content (TPC) was determined based on the reduction of FC reagent (phosphomolybdate and phosphotungstate) by phenolic compounds [16]. Gallic acid was used as a standard. Sample or gallic acid (20 µL, methanol) was added to 40 µL of 10% FC reagent (v/v, dH_2O) and mixed thoroughly (3 min). Sodium carbonate (160 µL, 7.5% w/v, dH_2O) was added and the mixture was incubated in dark condition for 2 h at room temperature (RT). The mixture absorbance was measured at 765 nm using microplate reader. TPC was expressed as milligrams of gallic acid equivalents, mg GAE/g extract). The total of phenolic content of sample (GAE mg/g extract) was calculated by the following formula:

$$\mathbf{C = c \times V / m}$$

Where; C = TPC of crude extracts (mg GAE/g extract); c = TPC of crude extracts obtained from gallic acid calibration curve (mg/mL); V = volume of extract (mL); m = weight of pure plant extract (g).

2.4. Total flavonoids content

Total flavonoid content was measured according to a modified protocol [17]. Quercetin was used as standard. Sample or quercetin (60 µL, diluted in methanol) was mixed with aluminium chloride (30 µL, 10% w/v), potassium acetate (30 µL, 1 M) and 52 µL dH_2O and incubated (30 min, RT). The absorbance was read at 415 nm. The total flavonoid content was expressed as milligram of quercetin equivalent per gram of dry sample (mg QE/g extract) as shown in equation below:

$$\mathbf{C = c \times V / m}$$

Where; C = TFC of crude extracts (mg QE/g extract); c = TFC of crude extracts obtained from gallic acid calibration curve (mg/mL); V = volume of extract (mL); m = weight of pure plant extract (g).

2.5. DPPH free radical scavenging assay

DPPH free radical scavenging assay was adopted with some modifications to determine DPPH free radical scavenging ability of wild edible vegetables [18]. Quercetin was used as standard reference. DPPH solution (200 µL, 0.125 mM in methanolic) was mixed with samples or quercetin (50 µL, 1 mg/mL in DMSO) and followed by two-fold dilution to obtain a series of concentrations. The mixture was incubated under dark condition (30 min, RT) and DPPH reduction was read at λ 517 nm against a blank (50 µL methanol with 200 µL DPPH solution) using microplate reader. The inhibitory concentration at 50% (IC_{50}) was determined from the graph of percentages inhibition versus extracts concentration ranging. Antiradical power (ARP) is defined as reciprocal of IC_{50}. DPPH scavenging activity and antiradical power were calculated using the following equations:

$$\text{Inhibition (\%)} = [(A_o - A_1)/ A_o)] \times 100$$

$$\text{Antiradical} = 1/IC_{50}$$

Where; A_o = absorbance of the control reaction; A_1 = absorbance of the sample itself.

2.6. Statistical analysis

The readings were measured in triplicate. Data was expressed as mean values ± standard error (SE)/ standard deviation (SD). Simple regression analysis was performed to discuss the relationships between antiradical capacity and polyphenols amount. All data collected were analysed using descriptive analysis in SPSS software version 20.0. Differences were considered significant at $p<0.05$.

3. Results

The results revealed that most of ethanolic freeze dried (EFD) samples contained higher phenolics composition (TPC and TFC) and better antioxidant potential compared to ethanolic oven dried (EOD). The highest TPC was determined in EFD of *T. catappa* with 65.55, while its EOD contained 48.33 (mg GAE/g extract). The least was found in EFD of *P. acidus* (17.51) and EOD of *E. hieraciifolia* (11.31) (mg GAE/g extract). For TFC, EFD and EOD of *O. indicum* contained the highest amount with 55.96 and 30.14, respectively, while EFD of *E. hieraciifolia* and EOD of *P. sarmentosum* had the least with 17.07 and 11.32, respectively (mg QE/g extract) (Fig. 1).

On the other hand, further study was carried out to compare the effect of extractant solvent towards polyphenols composition. The results revealed that the phenolics and flavonoids content as well as antioxidant activity of almost all ethanolic extracts (EFD) were

respectively higher and stronger than aqueous extracts (AFD). Among AFD samples, *T. catappa* contained higher amount of TPC (53.73 mg GAE/g extract) and TFC (41.51 mg QE/g extract) compared to others. The least amount of TPC was found in AFD of *E. hieraciifolia* (11.56 mg GAE/g extract), while for TFC, *P. sarmentosum* contained the least (2.25 mg QE/g extract) (Fig. 2).

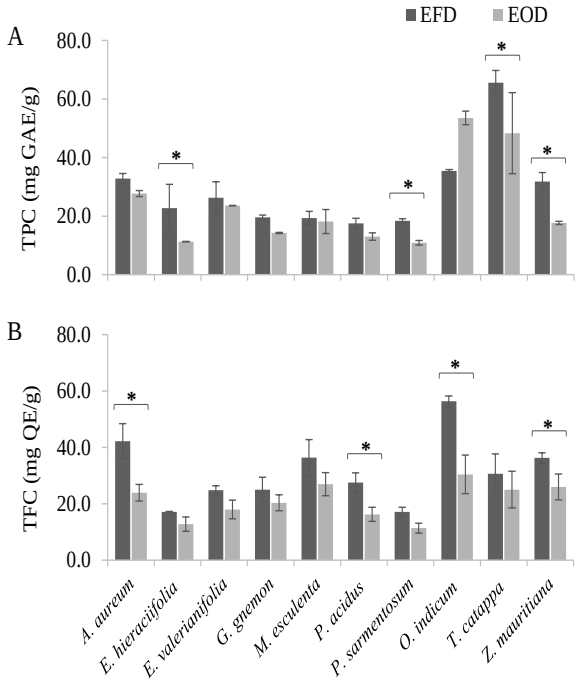


Fig. 1: Effect of drying methods on phenolics components in wild edible vegetables extracts. A) Total phenolics content (TPC), B) Total flavonoids content (TFC). *EFD = ethanolic freeze-dried extracts, EOD = ethanolic oven-dried extracts. Data represents means ± SD, n = 4. Differences were considered significant at: * = $p<0.05$.

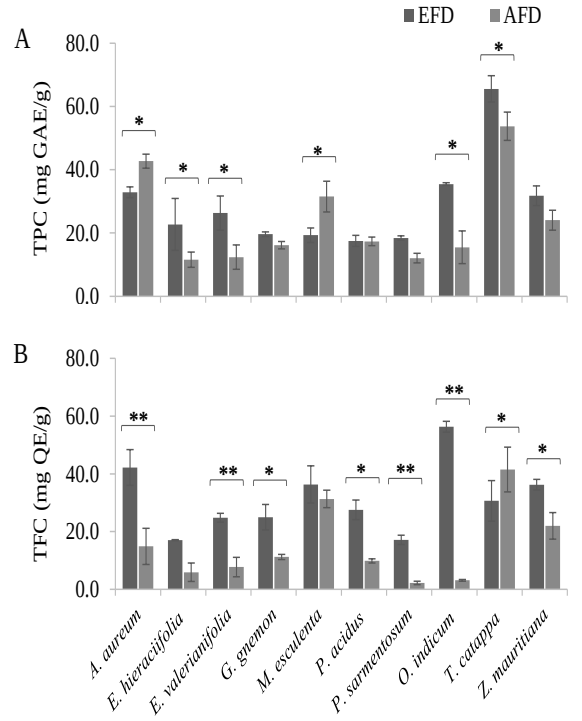


Fig. 2: Effect of extraction solvents on phenolics components in wild edible vegetables extracts. A) Total phenolics content (TPC), B) Total flavonoids content (TFC). *EFD = ethanolic freeze-dried extracts, AFD = aqueous freeze-dried extracts. Data represents means ± SD, n = 3. Differences were considered significant at: * = $p<0.05$, ** = $p<0.01$.

Furthermore, DPPH free radical inhibition by EFD samples at 0.5 mg/mL were in range of 76.1% to 88.1% (Fig. 3A), while EOD samples inhibited 56.9% to 87.9% (Fig. 3B). Among EFD samples, *O. indicum* and *T. catappa* was the most active with IC_{50} values 15.56 and 22.71 µg/mL, respectively. The weakest activity was displayed by *G. gnenom* (194.05 µg/mL). Similarly, among EOD samples, *O. indicum* and *T. catappa* indicated more antioxidant potential, while the least inhibition was also shown by *G. gnenom* (Table 1). Meanwhile, DPPH inhibition of AFD samples at 0.5 mg/mL were ranging from 35.2% to 89.4% with IC_{50} values from 6.19 µg/mL to 635.14 µg/mL (Fig. 3C). AFD of *T. catappa* demonstrated the strongerest antioxidant potential, while *E. hieraciifolia* was the weakest (Table 1).

3. Discussion

The recent study proved that most of samples following freeze-drying (FD) and ethanol extraction retained higher polyphenolics amount while displaying stronger antioxidant potential compared to oven-drying (OD). In the recent study, the antiradical power (ARP) calculated as reciprocal of IC_{50} of DPPH scavenging activity ($1/IC_{50}$) was used to define the antioxidant action of a sample antioxidant capacity. In addition, antioxidant capacity correlation with TPC and TFC were also investigated since the polyphenols have been discussed to contribute significant role in antioxidant activity [19].

These findings indicating more TPC (6.9 to 50.2%) and TFC (18.5 to 46.0%) in FD than OD samples correspond to the previous researches which suggested that FD preserve natural compounds by quenching metabolism via enzymes denaturation and microbial activities inhibition [20]. For example, freeze dried persimmon was reported to contain the highest total phenolics due to limited thermal and chemical degradation [9]. Generally, thermal treatments are known to contribute to the depletion of polyphenols in food products [12]. Additional of heat to heat-sensitive components such as polyphenols could lead to their loss due to enzymatic degradation by the Maillard reaction [21]. Thus, FD is often considered to be the most effective technique for preserving temperature sensitive compound. Besides, the samples ARP after undergoing OD thermal treatment was found to be reduced at about 33.6 to 92.0% compared to ARP of samples following FD. Therefore, FD is regarded as the better method for moisture removal in preserving metabolic features of herbs [7, 9].

The current study employed ethanol and aqueous as extractants due to the fact of its efficiency as good extractant of phenolic constituents while gave higher scavenging activity [22, 23]. Polar solvents are found to be effective to recover phenolics from plant samples. For examples, the highest amount of phenolics from barley flour were determined in ethanol/acetone extract [24] and lower molecular weight polyphenols were found to be extracted effectively using methanol, while bigger flavanols have high affinity with aqueous/acetone [25]. This study also revealed that most of ethanolic extracts recovered more TPC (17.7 to 53.3%) and TFC (13.9 to 94.4%) compared to aqueous extracts. In addition, ARP of ethanolic extracts also higher at about 33.7 to 94.6% than ARP of aqueous extracts. However, some aqueous extracts have more ARP (*A. aureum*, *M. esculenta* and *T. catappa*), TPC (*A. aureum* and *M. esculenta*) and TFC (*T. catappa*) compared to ethanolic extracts. This possibly due to the active compounds of different chemical properties and polarities may be soluble or insoluble in a certain particular solvent [13].

The presence of bioactive compounds in alcoholic *T. catappa* and *O. indicum* extracts are in agreement with the work of other researchers. Previous works also recorded that major polyphenols compounds in ethanolic extracts of *T. catappa* including phenolics, flavonoids, and tannins [26]. Meanwhile, *O. indicum* was also reported to be rich in flavonoids constituents [27, 28].

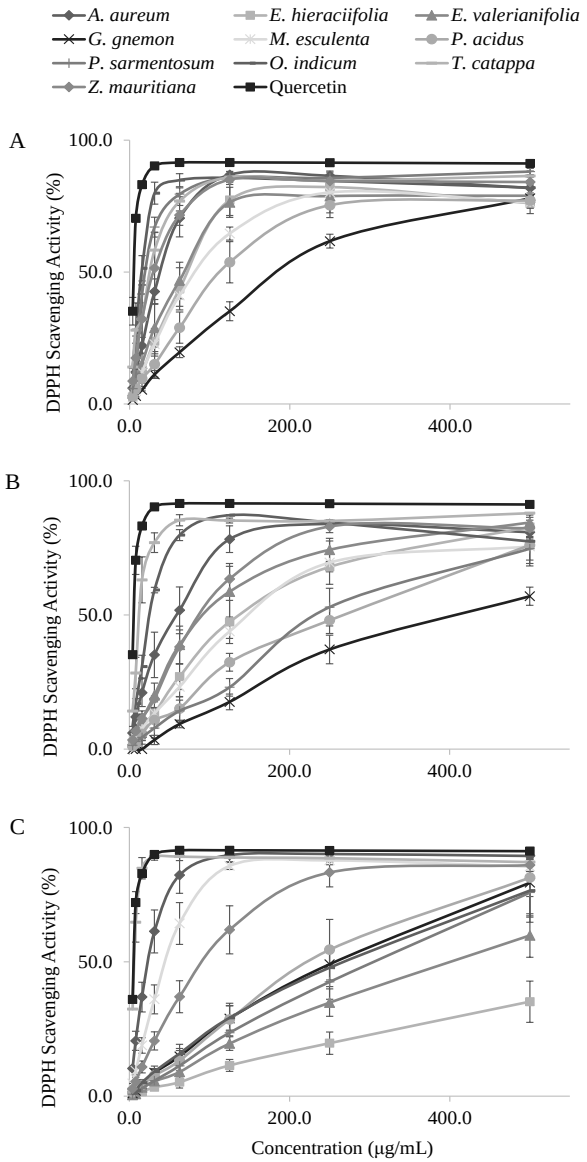


Fig. 3: DPPH free radical scavenging activity (%) of wild edible vegetables extracts at different concentration. A) Freeze-dried samples extracted with ethanol (EFD), B) Ovendried samples extracted with ethanol (EOD), C) Freeze-dried samples extracted with water (AFD). Data represents means ± SE, n = 3.

Table 1: IC_{50} values of DPPH radical scavenging activity of ethanolic and aqueous extracts of Malaysian wild edible vegetables after freeze and oven drying.

Vegetables	DPPH scavenging activity (IC_{50}; µg/mL)		
	EFD	EOD	AFD
A. aureum	39.13±5.02	58.88±16.46	25.67±6.71
E. hieraciifolia	66.79±5.18	143.87±27.03	635.14±15.34
E. valerianifolia	67.52±16.17	145.17±58.18	357.28±15.55
G. gnemon	194.05±14.59	405.75±51.83	292.71±93.81
M. esculenta	83.93±7.52	160.46±18.21	48.04±8.95
P. acidus	122.00±24.23	276.19±43.9	219.36±33.47
P. sarmentosum	20.79±2.08	260.24±23.44	325.33±79.26
O. indicum	15.56±1.70	23.45±2.05	285.92±77.08
T. catappa	22.71±3.47	10.26±4.45	6.19±0.87
Z. mauritiana	33.54±12.29	91.45±14.78	101.07±24.98

*EFD = ethanolic freeze-dried extracts, EOD = ethanolic oven-dried extracts, AFD = aqueous freeze-dried extracts. Data represents means ± SE, n = 4.

The antioxidant activities of *T. catappa* and *O. indicum* also could also attributable to other bioactive compounds such as reducing sugars, alkaloids, glycosides, tannins, flavonoids gums and terpenoid which also soluble in other polar solvents besides ethanol [29-31].

To discuss antioxidant capacity correlation with TPC and TFC, linear correlations between ARP and polyphenols were determined (Fig. 4). The TPC were found to has strong positive correlation with antioxidant activity with correlation coefficient, R was 0.7331 and determination coefficient, R^2 was 0.5374. However, a weak correlations were observed between antiradical and flavonoids content (R = 0.5302; R^2 = 0.2812). These are consistent with previous literatures that discussed the positive correlation between plants extracts antioxidant activity and phenolic compounds [19]. The polyphenols were reported to function as antioxidants due to their ability to donate electrons or hydrogen as well as react as stable radical intermediates [32].

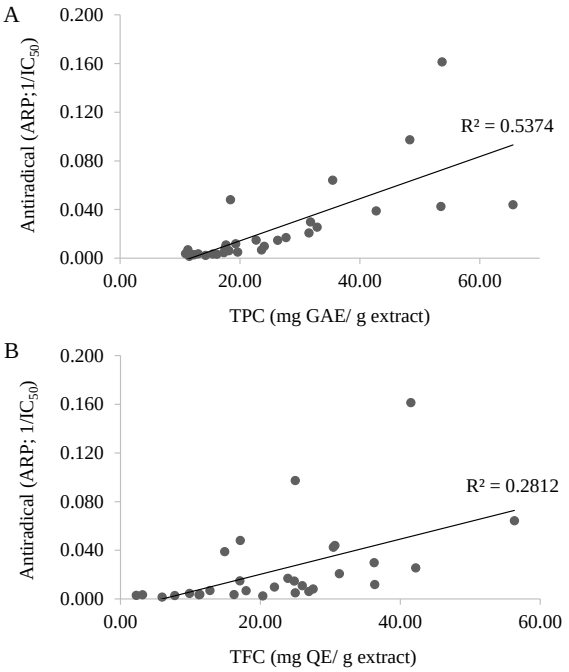


Fig. 4: Antioxidant capacity correlation with A) Total phenolics content (TPC) and B) Total flavonoids contents (TFC).

4. Conclusion

Most of the samples following freezed-drying and ethanol extraction were proved to retain higher polyphenolics amount while displaying stronger antioxidant potential. Interestingly, aqueous extracts of certain species showed more potential. Thus, specific methods could be explored to warrant retention of the highest amount of naturally occurring antioxidant active compounds from individual vegetable.

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