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EVALUATION OF PLANT EXTRACTS FOR SWEETNESS USING THE MONGOLIAN GERBIL[1]

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ABSTRACT.—Extracts of *Thladiantha grosvenorii* fruits, *Stevia rebaudiana* leaves, and *Abrus precatorius* leaves were investigated using Mongolian gerbil electrophysiological and conditioned taste aversion procedures, which were designed to respond to sucrose. A close correlation was observed between extracts of these sweet plants known to contain sweet principles and those extracts indicated as being sweet by a combination of these gerbil bioassays. The methods employed seem to be suitable for use in aiding the purification of highly sweet compounds of plant origin.

The plant kingdom has afforded many examples of substances that are highly sweet when compared to sucrose, especially compounds in the terpenoid, flavonoid, and protein structural classes (2,3). Several pure or partially purified plant constituents have approval as high-intensity sweetening agents, as exemplified by the current use of glycyrrhizin, mogroside V, phyllodulcin, stevioside, and thaumatin in Japan (4). In addition, neohesperidin dihydrochalcone and perillartine are examples of semisynthetic plant-derived constituents that are commercially available for sweetening purposes in one or more countries (5,6).

A crucial step in the isolation of novel potentially sweet compounds from plants is the assessment of the sweetness of extracts and chromatographic cuts of various polarities by one or more human volunteers (7). In a typical situation, there is no literature or anecdotal evidence suggesting that a given sweet-tasting plant part might also contain toxic substances. However, the aerial parts of *Lippia dulcis* Trev. biosynthesize the toxic monoterpene, camphor, in addition to the sweet sesquiterpene, hernandulcin (8), and *Hemsleya panicis-scandens* C.Y. Wu and Z.L. Chen rhizomes have been found to contain various cytotoxic cucurbitacins as well as the sweet cucurbitane glycoside, scandenoside R6 (9). Thus, as a precaution against the possibility of toxins co-occurring with sweet substances, we have routinely subjected extracts of sweet-tasting plant parts to bacterial mutagenicity evaluations and acute toxicity tests in mice prior to their being tasted for sweetness (10–13). However, this practice is both time-consuming and expensive. Therefore, in an attempt to avoid the need for human involvement in assessing the sweetness of plant extracts, we have investigated alternative methodology that utilizes a combination of established electrophysiological and behavioral procedures to determine gustatory stimulation in the Mongolian gerbil, *Meriones unguiculatus*.

It has been shown previously that the gerbil's intact chorda tympani nerve is stimulated by sweet monosaccharides, disaccharides, and polyols, in addition to many naturally occurring and synthetic intensely sweet substances such as chlorosucrose, L-cyanosuccinanilic acid, dulcin (*p*-ethoxyphenylurea), sodium saccharin, stevioside, and D-tryptophan

[1]Paper No. XXIII in the series "Potential Sweetening Agents of Plant Origin." See Hussain *et al.* (1) for part XXII.

(14–17). The method is logistically capable of being applied on up to 25 samples per day, and is highly sensitive to some sweeteners, as in the case of stevioside, which has been recorded as being stimulatory when present in a concentration as low as 2×10^{-5} M (24 μg/ml) (17). A behavorial conditioned taste aversion test (CTA) may then be used to characterize the taste of electrophysiologically active samples, in which the degree of similarity of taste is determined by the amount of experimental fluids consumed by gerbils trained to avoid sweet, salty, bitter, and sour taste qualities. Animals may be used in such behavioral experiments for as long as 9 months once they have been trained (17–19).

In the present preliminary study, extracts were prepared from three sweet-tasting plants: the fruits of *Thladiantha grosvenorii* (Swingle) C. Jeffrey (*syn. Momordica grosvenorii* Swingle) (Cucurbitaceae) and the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae) and *Abrus precatorius* L. (Fabaceae). These species contain highly sweet glycosides of the cucurbitane (e.g., mogroside V), *ent*-kaurene (e.g., stevioside, rebaudioside A), and cycloartane (e.g., abrusosides A–D) types, respectively (2,3,13). Thirteen extracts of varying polarities were prepared from the three plants, and the results of gerbil electrophysiological and behavioral testing on these extracts are expressed in Tables 1 and 2.

It may be seen from Table 1 that all of the plant extracts tested produced electrophysiological responses. Using this method, concentrations were normally determined which produced a stimulatory response equivalent to that of 0.03 M sucrose, with the exception of several extracts that exhibited limited solubility. The *T. grosvenorii* 80% MeOH and H_2O extracts, the *S. rebaudiana* 80% MeOH, *n*-BuOH, and H_2O extracts, and the *A. precatorius n*-BuOH and H_2O extracts were all found to be effective, with such extracts ranked in the order of potency, *S. rebaudiana* > *T. grosvenorii* > *A. precatorius*. These data accurately reflect the approximate yields of the sweet principles of the three plants investigated: *S. rebaudiana* (*ent*-kaurene glycosides, ca. 10–15% w/w), *T. grosvenorii* (cucurbitane glycosides, ca. 1% w/w), and *A. precatorius* (cycloartane glyco-

TABLE 1. Summary of Gerbil Electrophysiology Experiments Using Plant Extracts.

Plant extract	Maximum concentration tested (mg/ml)	Number	Sucrose (0.03 M) response (%)
Thladiantha grosvernorii fruits			
80% MeOH	5	9	112
Et_2O	5[a]	6	34
n-BuOH	5[a]	8	48
H_2O	6.3	10	136
Stevia rebaudiana leaves			
80% MeOH	2	10	96
Et_2O	5	9	40
n-BuOH	2	10	87
H_2O	2	10	108
Abrus precatorius leaves			
80% MeOH	2[a]	8	52
Petroleum ether	5[a]	9	21
Et_2O	5[a]	9	6
n-BuOH	10	9	50
H_2O	10	9	66

[a]Difficult to get into solution.

TABLE 2. Comparison of Gerbil Gustatory Behavior with Known Sweetener Content of Plant Extracts ($N = 12$ animals per group).

Plant extract	Concentration (mg/ml)	Mean intake (± 2 SE) by CTA[a] group (ml)				H_2O (control)	Presence or absence of intense sweetener(s) in extracts
		Sucrose	Quinine	HCl	NaCl		
Thladiantha grosvernorii fruits							
80% MeOH	5	0.42[b] ± 0.22	1.09[b] ± 0.44	1.08 ± 0.56	1.67 ± 0.38	1.70 ± 0.44	Present[c]
Et_2O	5	0.90 ± 0.24	0.61[b] ± 0.20	1.31 ± 0.40	1.46 ± 0.29	1.20 ± 0.32	Absent
n-BuOH	5	1.68 ± 0.40	2.04 ± 0.60	2.43 ± 0.38	2.44 ± 0.36	2.21 ± 0.33	Absent
H_2O	6.3	0.28[b] ± 0.02	1.09[b] ± 0.41	1.90 ± 0.32	1.83 ± 0.74	1.60 ± 0.40	Present[c]
Stevia rebaudiana leaves							
80% MeOH	2	0.32[b] ± 0.02	0.67[b] ± 0.42	1.59 ± 0.42	1.37 ± 0.54	1.85 ± 0.32	Present[d]
Et_2O	5	0.38 ± 0.10	0.47 ± 0.10	0.53 ± 0.14	0.72 ± 0.37	0.53 ± 0.30	Absent
n-BuOH	2	0.31[b] ± 0.22	0.46[b] ± 0.38	1.12 ± 0.32	1.16 ± 0.37	1.00 ± 0.42	Present[d]
H_2O	2	0.29[b] ± 0.08	0.73[b] ± 0.28	1.54 ± 0.32	1.63 ± 0.26	1.64 ± 0.26	Present[d]
Abrus precatorius leaves							
80% MeOH	2	1.28 ± 0.59	2.07 ± 0.70	1.68 ± 0.58	1.90 ± 0.54	2.00 ± 0.40	Present[e]
Petroleum ether	5	1.56 ± 0.48	1.35 ± 0.55	1.50 ± 0.90	1.50 ± 0.74	1.92 ± 0.44	Absent
Et_2O	5	1.28 ± 0.34	1.12 ± 0.35	1.70 ± 0.35	1.51 ± 0.31	1.32 ± 0.30	Absent
n-BuOH	10	0.31[b] ± 0.01	0.67 ± 0.32	1.16 ± 0.20	0.99 ± 0.40	0.81 ± 0.34	Present[e]
H_2O	10	1.13[b] ± 0.32	1.35 ± 0.41	1.96 ± 0.34	1.71 ± 0.34	1.54 ± 0.26	Present[e]

[a]Conditioned taste aversion.
[b]Significant ($p < 0.05$).
[c]Cucurbitane-type triterpenoid glycosides.
[d]*ent*-Kaurene-type diterpene glycosides.
[e]Cycloartane-type triterpene glycosides.

sides, ca. 0.4% w/w) (2,3,13,20). The remaining extracts from the three plants were not regarded as stimulatory using this assay.

When the gerbil CTA studies performed on the four *T. grosvenorii* fruits are considered (Table 2), gerbils trained to avoid sucrose solution drank less of the 80% MeOH and H_2O extracts, which are the only ones that would contain the highly polar triterpene glycoside, mogroside V, the principal sweet constituent of this species (2,3,20). Those gerbils trained to avoid quinine drank less of the *T. grosvenorii* 80% MeOH, Et_2O, and H_2O extracts, when compared with controls. In the case of the *S. rebaudiana* extracts, sweet *ent*-kaurene glycosides are partitioned from the 80% MeOH extract into both the *n*-BuOH extract (dulcoside A, rebaudiosides A–C, steviolbioside, stevioside) and the H_2O extract (rebaudiosides D and E). No sweet principles would be expected to partition into the Et_2O layer (2,3,21). Gerbils trained to avoid sucrose drank less of these three extracts, but not less of the Et_2O extract, than controls. In addition, it was observed that gerbils trained to avoid quinine drank less of the 80% MeOH, *n*-BuOH, and H_2O extracts of *S. rebaudiana* relative to controls (Table 2). Stevioside and the several of the other *S. rebaudiana* sweet principles (especially rebaudioside C) are known to elicit bitter tastes in humans, along with their sweetness (2,3). For the *A. precatorius* extracts, only the *n*-BuOH and H_2O extracts were drunk to a lesser extent by gerbils trained to avoid sucrose (Table 2). Abrusosides A–D, the triterpene glycoside constituents responsible for the sweetness of this species, each possess one or more carboxylic acid units, and would thus be expected to partition into *n*-BuOH when in the free acid form and into H_2O when in the form of salts with one or more cations (13). The slightly sweet-tasting *A. precatorius* 80% MeOH extract (13) was not drunk to a lesser degree than controls by animals trained to avoid sucrose, although this was tested at a lower dose than the *A. precatorius* *n*-BuOH and H_2O extracts (Table 2).

Therefore, the data expressed in Tables 1 and 2 show that good correlations were evident between the results of the gerbil experiments and the presence or absence of sweetness of the extracts represented, for all three plants studied. Had the sweet principles of *T. grosvenorii*, *S. rebaudiana*, and *A. precatorius* never been investigated before, then a combination of these gerbil electrophysiological and behavioral studies would have indicated correctly the preferential solubility of their sweet constituents. While the present preliminary investigation suggests that this gerbil methodology may hold the potential for wider application for the screening of plant-derived extracts and fractions for sweetness, several limitations must be stated. First, problems were encountered in the solubilization of the nonpolar plant extracts. Second, the gerbil's chorda tympani nerve only innervates the tip of the tongue, although this is very similar to the tip of the tongue of humans and responds well to sweeteners. Third, the gerbil is not a perfect model for human sweet taste perception, because some sweeteners such as aspartame and monellin do not stimulate its chorda tympani nerve, while other substances such as sodium cyclamate do not resemble the taste of sucrose in CTA studies. For example, in a previous study, 14 out of 21 non-saccharide sweet substances were found to produce neural responses in gerbil electrophysiological experiments, of which the majority were later assessed as being "sweet," "sweet-salty," or "sweet-bitter" on subsequent CTA experiments (17). Overall, however, the gerbil assays should prove useful, because more compounds compare favorably with the human sweet taste behavior than those that do not. The gerbil electrophysiological assessment of several pure naturally occurring highly

sweet compounds of plant origin is under way.

EXPERIMENTAL

PLANT MATERIAL.—Authentic samples of the fruits of *T. grosvenorii*, the leaves of *S. rebaudiana*, and the leaves of *A. precatorius* were obtained as described in earlier publications (13,20,21). Voucher specimens have been deposited in the herbarium of the Field Museum of Natural History, Chicago (13,20,21).

EXTRACTION PROCEDURE.—Powdered *T. grosvenorii* fruits (50 g) were percolated three times with 80% MeOH to yield 14.6 g of a brown gum on removal of solvent under reduced pressure. A portion of this dried 80% MeOH extract (2.98 g) was retained for testing in the present investigation, and a further 6.0 g was then sequentially dissolved in aliquots of Et_2O (3 × 100 ml), *n*-BuOH (3 × 100 ml), and H_2O (3 × 25 ml), to afford, on removal of solvent, dried Et_2O (0.83 g), *n*-BuOH (0.54 g), and H_2O (2.34 g) extracts. A similar procedure was employed to generate dried 80% MeOH (1.03 g), Et_2O (0.5 g), *n*-BuOH (1.0 g), and H_2O (1.39 g) extracts of *S. rebaudiana* leaves. In the case of *A. precatorius* leaves, the initial 80% MeOH extract was dissolved in petroleum ether (3 × 100 ml) prior to partition into Et_2O, *n*-BuOH, and H_2O. The weights of the resultant dried residues used for testing were petroleum ether (2.98 g), Et_2O (2.52 g), *n*-BuOH (2.82 g), and H_2O (2.36 g).

EXPERIMENTAL ANIMALS.—Male Mongolian gerbils (*M. unguiculatus*), each weighing ca. 50 g, aged 7–12 weeks, were purchased from Tumblebrook Farm, West Brookfield, Massachusetts.

ELECTROPHYSIOLOGICAL EXPERIMENTS.—Electrophysiological recordings on plant extracts completely dissolved or dissolved to the greatest extent practicable in distilled H_2O were obtained from the chorda tympani nerves of anethestized gerbils according to an established procedure (14–17). Each extract was dissolved in hot deionized H_2O up to about saturation and cooled to about 25° before testing. Upon cooling, the nonpolar extracts formed milky emulsions that were used in the experiments. The integrated nerve discharge was used to characterize the responsiveness of the gerbil's anterior tongue receptors to each plant extract. Typically, up to 10 replicate measurements were made at the concentration of each extract that produced a stimulatory response equivalent to that produced by 0.03 M sucrose.

BEHAVIORAL EXPERIMENTS.—Mongolian gerbils were used in behavioral experiments only when previously subjected to H_2O intake training and conditioned avoidance training. The following procedures were used in these conditioned taste aversion experiments.

H_2O intake training.—Upon arrival, the animals were housed in individual wire bottom cages and were placed in a drinking schedule whereby they received deionized H_2O twice a day from 9.00–10.00 a.m. and from 3.00–4.00 p.m. Animals were fed Purina Rat Chow® ad libitum.

After 2 weeks, the average daily H_2O intake of each gerbil was measured, and the "heavy" and "light" drinkers were taken from the group. While it was found that most of the animals consumed about 3.0 ml of H_2O per day, the intake of the so-called heavy drinkers was >5.0 ml. The so-called light drinkers consumed little or no H_2O during the training period and tended to lose weight from dehydration.

Conditioned avoidance training.—After H_2O intake training, the animals were divided into five groups of twelve, of which one group was trained to avoid sucrose (sweet), one group trained to avoid quinine (bitter), one group trained to avoid HCl (sour), one group trained to avoid NaCl (salty), and one control group trained to avoid H_2O. A sixth group, of matched weight and the same H_2O intake, was maintained on the normal drinking schedule and used to replace injured members of the avoidance groups. From past experience (17–19), these included animals with excessive weight loss, weight gain, and/or hair loss.

The solutions used in the conditioning procedure [0.03 M sucrose; 0.001 M quinine (as HCl salt); 0.01 M HCl; 0.1 M NaCl] were provided each of 2 consecutive weeks. Thus, during the normal morning drinking period, the five groups were offered bottles containing either sucrose, quinine, HCl, NaCl, or distilled H_2O for 5–10 min. As soon as each animal finished drinking, its drinking tube was again placed in its mouth, leaving a few drops behind, and immediately thereafter, the gerbil was injected with 0.3 M LiCl (at 1% of its body wt). Shortly after such an injection, each animal demonstrated a lethargic appearance that lasted for several hours. This entire avoidance training procedure was repeated the following Friday.

Conditioned avoidance testing.—On Monday of the third week, having allowed 2 days for the recuperation of the animals subjected to conditioned avoidance training, half the animals in each group were offered H_2O bottles containing one of two unknowns. Then, the following morning, the animals in each group received the unknown that was not offered the day previously. Measurements of the amounts of fluids consumed were made by weighing the drinking bottle immediately after each animal stopped drinking. During the afternoon drinking periods, all animals received only deionized H_2O. Testing was

continued in this manner until all of the unknown samples had been studied. The conditioning procedure was repeated on each Friday morning for the length of the overall study, in order to maintain gerbils with the same level of avoidance as previously.

The concentration of each plant extract used in the behavioral CTA studies was that which produced an electrophysiological response equivalent to 0.03 M sucrose. The similarity of taste was determined by the amount of each plant extract drunk by animals trained to avoid sweet (0.03 M sucrose), bitter (0.001 M quinine), sour (0.01 M HCl), and salty (0.1 M NaCl) tastes. In these assays, fluid intakes were first compared statistically by ANOVA and then by *t*-test, whenever a statistical interaction was observed (17–19).

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