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DIPHENYLAMINE, AN ANTIHYPERGLYCEMIC AGENT FROM ONION AND TEA

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ABSTRACT.—Diphenylamine has been isolated as one of the active antihyperglycemic agents of onion. It was identified by ir, uv, cmr, pmr, and mass spectra. It attained the highest percentage in mature onion bulbs; other onion organs contained diphenylamine in lower percentages. Green and black teas were found to contain relatively high percentages of diphenylamine, being higher in the former. Cooking was found to decrease the diphenylamine content.

Antihyperglycemic agents, especially those of plant origin, have received much attention recently. Many plants and plant products (1) were claimed to have certain beneficial effects on diabetes in folk medicine. Onion (2) and a number of its extracts (3) have been reported to possess hypoglycemic activity. Allylpropyldisulfide, a component of the volatile oil of onion, has been found to produce hypoglycemia and to effect a rise in serum insulin in normal fasting human subjects (4). Several reviews have appeared on the subject (5-8).

Upon the oral administration of crude onion juice reduction of the blood-sugar level, in cases of induced hypoglycemia, has been reported, both in laboratory animals and in human volunteers (9-11). These observations support the presence of a potent hypoglycemic principle in onion, which has not been isolated previously. Mathew and Augusti (12), Augusti (13), and Georgy (14) have pointed out that such antihyperglycemic agents are chiefly extracted with Et_2O.

Tea, the most popular hot drink in Asia, Africa, and Europe, has also been claimed to possess a hypoglycemic activity (15). In the course of an investigation of tea flavor, Nose *et al.* (16) identified diphenylamine as one of the major constituents in green tea leaves, using gc-ms. Diphenylamine has also been identified in citrus fruit rind oils by *gc* (17).

The present study (18) is directed towards the isolation and identification of the antihyperglycemic agent(s) of onion and tea.

RESULT AND DISCUSSION

The petroleum ether extract, the Et_2O extract, and the $CHCl_3$ extract produced a marked reduction in the blood-sugar level of 50.6, 58.0, and 33.7 percent, respectively. Thus, the maximum hypoglycemic activity was contained in the Et_2O extract. These findings coincide with those reported by Augusti (13) and Mathew and Augusti (12), while Gupta *et al.* (9) stated that the $CHCl_3$ extract was most effective. The latter findings, however, do not seem contradictory, since Gupta *et al.* utilized $CHCl_3$ before Et_2O in the order of extraction.

The petroleum ether-soluble fraction of the Et_2O extract of onion was chromatographed on one of two columns: either silica gel/*n*-heptane-diisopropyl ether (1:1) or alumina/isooctane.

Diphenylamine fractions from either column [tlc, Rf 0.82 on silica gel G/*n*-heptane-diisopropyl ether (1:1)] were pooled, evaporated to dryness, and crystallized from Et_2O to give white crystals, mp 53°-55°, which were optically inactive and possessed a molecular formula of $C_{12}H_{11}N$, as shown from elemental analysis.

Spectral evidence was in support of the structure of diphenylamine for the isolated compound. The uv spectrum showed λ max at 286 and 241 nm. The ir spectrum showed peaks at ν NH 3370, ν C-H (aromatic) 2900, 3000 and ν (monosubstituted benzene) at 860, 740, 690, and 680 cm^{-1}.

Pmr showed a broad singlet at 5.8 ppm (1 H-broad singlet) corresponding to -NH and a symmetric multiplet between 6.8 and 7.5 ppm (10 H-multiplet) corresponding to ten aromatic protons.

The cmr of the compound (Table 1) showed only four peaks, one of them due to quaternary carbon(s). The simplicity of the spectrum indicated the symmetric structure of the isolated compound. Calculated ^{13}C-chemical shifts for diphenylamine (19) are in good agreement with the found values.

TABLE 1. Cmr of Diphenylamine

Carbon No.	Chemical Shift (ppm)	
	Found value	Calculated value[a]
C, 1, 1′	143.2	144.8
C, 2, 2′, 6, 6′	117.9	124.1
C, 3, 3,′ 5, 5′	129.3	129.1
C, 4, 4′	121.0	122.6

[a]According to Pretsch *et al.* (19).

The mass spectrum of the isolated compound showed a molecular ion peak (M^+) at *m/z* 169 (100%, base peak) and corresponded in all details with those reported in the literature for diphenylamine (16).

In view of this compound being an unusual plant constituent, the assignment of structure as diphenylamine was confirmed by undepressed mmp with authentic material, cochromatography, and superimposable ir spectra with authentic diphenylamine. Further, both isolated and authentic material reacted with HCl in the presence of HNO_3, turning blue (20).

When tested for hypoglycemic activity in hyperglycemic rabbits[1] (Table 2),

TABLE 2. Average Blood-Sugar Level (mg %)[a] after Administration of Isolated Substance as Compared to Tolbutamide

Drug	Fasting	After oral glucose load (2.25 g/kg body weight)					% Reduction at 2 h
		½ h	1 h	1½ h	2 h	2½ h	
Control	102.0 ±5.1	162 ±4.1	180 ±15.4	182.2 ±14.2	186 ±12.1	173.0 ±9.2	
Diphenylamine . . . . . 10 mg/kg body weight	102.2 ±0.86	140.2 ±7.8	154.6 ±1.72	80.2 ±2.6	71.8 ±6.2 P<0.0005	77.2 ±5.4 P<0.0005	61.39
Tolbutamide . . . . . 50 mg/kg body weight	104.2 ±2.9	129.0 ±3.5	154.3 ±10.1	125.2 ±6.2	104.0 ±3.1 P<0.0005	95.1 ±3.4 P<0.0005	44.08

[a](mean±SE). Statistical tests used were standard error of the mean and Student's *t* Test, according to J.D. Hinchen, "Practical Statistics for Chemical Research," London: Methuen & Co., Ltd., 1969.

[1]Glucose was administered as 45% w/v solution through a stomach tube 30 min prior to the ingestion of the required extracts.

diphenylamine in 10 mg/kg body weight caused a 61.4% ($\pm 3.3, p<0.0005$) reduction in blood sugar 2 h after oral administration of the drug. Comparatively, tolbutamide in a dosage of 50 mg/kg body weight caused 44.1% ($\pm 1.65, p<0.0005$) reduction in the blood sugar, 2 h after administration. Thus, diphenylamine is a more potent antihyperglycemic agent than tolbutamide in this test system.

The quantitative estimation of diphenylamine in the plant material was successfully achieved spectrophotometrically where Beer's law was obeyed over a concentration range of 2-12 μg/ml in EtOH at 286 nm. This method compared well with a fluorimetric method that was also adopted using excitation and emission wavelengths at 300 nm and 400 nm, respectively, and was suitable for measuring diphenylamine over a concentration range of 2-16 μg/ml.

The determination was done on different varieties of onion and in different organs. The effect of maturity stage and of drying and cooking on diphenylamine content was also studied (Table 3). Mature onion scales and other organs of onion are very poor in this substance. Drying and cooking were found to decrease the diphenylamine content. Such decrease is much more pronounced in the case of drying, which decreased the content to 0.0005%. Cooked onion contained between 0.011 and 0.05% diphenylamine. Such decrease may be attributed to sublimation (drying) and distillation (cooking), since diphenylamine was found to sublime and to distill with steam. In the case of onion and vinegar salad, the vinegar was found to contain >85% of the diphenylamine content, while only ~9.0% remained in the onion slices. This may be due to its solubility in the acetic acid medium (vinegar).

In the course of a study of the tea flavor, Nose *et al.* (16) reported the presence of diphenylamine in green tea by gc/ms. They claimed black tea was devoid of this compound. Accordingly, many products of tea available in the Egyptian market have been investigated for diphenylamine content (Table 3). The occurrence of this compound in green and black teas could be confirmed by its isolation; the highest percentage (1.95%) of diphenylamine occurred in green tea, while high grades of black tea contained up to 1.17% and low grades as low as 0.4%. Investigation of the tea drink proved that only a small fraction of the diphenylamine content (1.1-2.25%) was released in the infusion, while 90-95% remained in the marc, either as the water-insoluble free amine or as its tannate. From the above, it is clear that the diphenylamine can be considered as one of the chief hypoglycemic agents in onion and tea. In the case of the tea, the activity remains chiefly in the marc.

EXPERIMENTAL

PLANT MATERIAL.—*Allium cepa* L. bulbs (onion), Giza 6, Giza 20, Beheri, and Early Grano varieties.[2] Various organs of onion studied were of Beheri variety, including seeds, green onion sprouts, immature bulbils, dry scales,[2] and dried onion slices and powder.[3] Cooked onion samples included boiled, fried, grilled, and pickled onions, as well as onion salad.

Tea samples included Yakoot Tea,[4] Blue Tea-Pot Brook Bond Tea,[5] Brook Bond Red Label Fannings,[5] Brook Bond Green Label Tea,[5] and Green Japanese Tea. These were obtained from the local market.

PREPARATION OF ONION EXTRACTS.—Two kg of onion were deprived of their outer membraneous scales, cut into small pieces and minced thoroughly in a blender. The pulp was strained through a multilayered muslin cloth. The filtrate was separated, and the marc was triturated in a mortar with a little distilled H_2O, filtered, and the marc discarded. The aqueous filtrate was subjected to exhaustive successive

[2]Courtesy of "Onion Research Section," the Agricultural Research Center, Ministry of Agriculture, Cairo, Egypt.

[3]El-Nasr Company for the Dehydration of Agricultural Products, Sohag, Egypt.

[4]Egyptian Co. for Packaging and Distribution of Foods, Cairo, Egypt.

[5]Brook Bond India Limited, Bombay, India, imported through Misr Import and Export Co., Cairo, Egypt (All grades) and Liebig Exports Limited, Gloucester, England (Green Label).

TABLE 3. Diphenylamine Content[a] in Onion and Tea

Onions (by variety)		Onions (by variety procedure)						Tea (by variety)	
		Cooked (Beheri)		Dried (Giza 6)		Organs (Beheri)			
Plant material	Diphenylamine (%)	Plant material	Diphenylamine (%)	Plant material	Diphenylamine (%)	Plant material	Diphenylamine (%)	Plant material	Diphenylamine (%)
Beheri	1.1	Boiled onion	0.022	Dried powder	0.0014	Mature bulb	1.1	Japanese green tea	1.95
Giza 6	0.89	Grilled onion	0.036	Dried Slices[b]	0.004	Immature bulb	0.53	Yakoot tea	0.4
Giza 20	0.79	Fried onion	0.052	Shade dried	0.004	Green leaves	0.059	Red label Brook Bond Tea	0.6
Early Grano (American)	0.92	Pickled onion	.025	Sun dried	0.003	Onion scales	0.0042	Green label Brook Bond Tea	0.94
		Onion+vinegar salad:		Oven dried	0.006	Onion seeds	0.051	Blue Tea-Pot Brook Bond Tea	1.17
		vinegar macerate	0.92						
		onion slices	0.011					Tea Drink	
								Drink	0.013-0.02
								Marc	1.066-1.14
								(Blue Tea-Pot brand)	

[a]Average of three determinations.
[b]El-Nasr Co. for Dehydration of Agricultural Products, Sohag, Egypt.

liquid-liquid extraction in a separator, using petroleum ether (40°-60°), Et_2O, and $CHCl_3$, respectively. The organic solvent extracts were separately distilled under reduced pressure and dried to a constant weight. We checked the blood-sugar lowering effect of the prepared onion extracts on induced hyperglycemia in rabbits, using the method recommended by Varley (21).

ISOLATION OF DIPHENYLAMINE.—The dried residue (4.7 g) left after the evaporation of the Et_2O extract was exhaustively extracted with petroleum ether. The petroleum ether-soluble fraction was evaporated under reduced pressure. The residue (1.71 g) was redissolved in the least amount of Et_2O and divided into halves. One half was submitted to chromatography on a column (60×3 cm) of neutral alumina using isooctane as the eluent. The other half was applied on a similar column containing silica gel using *n*-heptane mixed with increasing amounts of diisopropyl ether (0-3%) as eluent. In either case, 20-ml fractions were collected at a rate of 1.8 ml/min. Fractions from either column were subjected to tlc [silica gel G/diisopropyl ether-*n*-heptane (1:1)]; visualization was performed with *p*-dimethylaminobenzaldehyde-HCl spray. Fractions 20-32 of the alumina column revealed only one spot (Rf 0.82) which became yellow upon spraying, changing to greenish blue when heated at 110° for 10 min. These fractions were pooled and subjected to crystallization as mentioned below. Similarly, fractions of the silica column corresponding to a *n*-heptane-diisopropyl ether (7:3) eluent showed one spot (Rf 0.82); these were combined and the solvent was evaporated in vacuo. Crystallization was done from Et_2O, where fine white crystals, (0.95 g, mp 53°-55°) separated from the fractions obtained from both columns, $[\alpha]_D = 0°$ (EtOH). Elemental analysis, found: C, 85.25; H, 6.40 and N, 8.35%; cald. for $C_{12}H_{11}N$: C, 85.17; H, 6.55 and N, 8.28%; uv λ max 241 and 286 nm (log ϵ=41.028); ir ν 3370, 3000, 2900, 1600, 1520, 1500, 1420, 1330, 1180, 860, 740, 690, and 680 cm^{-1}; pmr ($CDCl_3$) δ (ppm) 5.8 ppm (1 H, broad s) and 6.8-7.5 ppm (10 H. multiplet); cmr (see Table 1); ms *m/z* 169 (M^+, 100%), 168 (65%), 167 (39%), 142 (4%), 141 (2%), 84 (18%), 78 (4%), 77 (16%), 66 (16%), and 51 (15%).

The isolated material was identical to authentic diphenylamine in every respect, mmp, cochromatography, superimposable ir spectra, and color reaction with HCl-HNO_3 (20).

EFFECT OF DIPHENYLAMINE ON BLOOD SUGAR LEVEL.—Diphenylamine, 10 mg/kg body weight, from both the isolated and synthetic compound, was administered separately to each of six healthy boskat rabbits through the oral route. A group of six rabbits was used as the control; each animal was fed with distilled H_2O only, through a stomach tube. These animals (test and control) were previously rendered hyperglycemic by giving them (2.25 g) glucose/kg body weight orally 30 min before the administration of the drug (test group) or of the H_2O (control). The blood-sugar lowering effect was compared with that of tolbutamide in a dose of 50 mg/kg body weight (Table 2).

The glucose level was determined by the Folin-Wu-Varley method (21). The method is based on alkaline $CuSO_4$ oxidation of the deproteinated blood filtrate, treating cuprous oxide obtained with tungstophosphomolybdic acid solution, and measuring the blueness obtained colorimetrically, comparing it with that of a standard glucose solution.

SPECTROPHOTOMETRIC DETERMINATION OF DIPHENYLAMINE IN DIFFERENT ONION VARIETIES, ORGANS, AND IN DIFFERENT FORMS OF COOKED ONION.—Of each sample 100 g was separately extracted with Et_2O and analyzed, adopting the spectrophotometric method. Quantitative, preparative isolation of the diphenylamine was done by tlc using silica gel and *n*-heptane-diisopropyl ether (1:1). The diphenylamine spot in each case was scraped off, eluted with EtOH (8 ml), the final volume adjusted to 10 ml, and its absorbance measured at 286 nm. A blank experiment was done using an area of silica gel free from solute, having the same Rf and the same area as the diphenylamine spot. The diphenylamine content was calculated from a constructed standard calibration curve, and the results are shown in Table 3.

FLUORIMETRIC DETERMINATION OF DIPHENYLAMINE IN DIFFERENT VARIETIES AND ORGANS OF ONION AND IN DIFFERENT FORMS OF COOKED ONION.—Of each sample, 100 g was separately extracted with Et_2O. Quantitative, preparative isolation of diphenylamine was done by tlc using Silica gel and *n*-heptane-diisopropylether (1:1). The diphenylamine spot in each case as detected by uv, was quantitatively scrapped off by a zone-extractor, eluted exhaustively with EtOH (8 ml), and the final volume adjusted to 10 ml. A control plate, free from diphenylamine load, was simultaneously developed in the same jar and blank areas corresponding to the Rf of diphenylamine were treated the same way and used as the blank. Each ethanolic eluate was determined in the fluorimeter against the prepared blank using excitation and emission wavelengths at 300 nm and 400 nm respectively. The diphenylamine content was calculated from a constructed standard calibration curve. The results compared well with the results obtained by the spectrophotometric method (Table 3).

DETERMINATION OF DIPHENYLAMINE IN ONION AND VINEGAR SALAD.—A sample of onion bulb (100 g) was cut into small pieces, macerated in a porcelain dish with 50 ml vinegar (6 v/v HOAc and allowed to stand for 3 h. The vinegar was filtered. The filtrate was made alkaline with 10% NaOH and extracted with Et_2O (3×50 ml) to exhaustion.

The marc was blended in a blender, filtered, and then exhausted with Et_2O (3×50 ml). The two Et_2O extracts obtained above were analyzed for diphenylamine using the above mentioned tlc-uv spectrophotometric or tlc-fluorimetric method.

ISOLATION OF DIPHENYLAMINE FROM TEA.—The same isolation procedure as described above for onion was adopted for the tea sample. It was recrystallized from Et_2O and found identical in every respect (mp, mmp, Rf, and ir) with diphenylamine isolated from onion as well as with authentic diphenylamine.

DETERMINATION OF DIPHENYLAMINE IN DIFFERENT BRANDS OF TEA.—A 50 g sample of each of the available brands of tea was percolated separately with Et_2O to complete exhaustion; each percolate was then evaporated at 30° under reduced pressure. Aliquots of each of the obtained extracts corresponding to 100 μg dried extracts were spotted on silica gel layers and assayed by the spectrophotometric or by the fluorimetric method as previously described. The results are shown in Table 3.

DETERMINATION OF DIPHENYLAMINE IN TEA DRINK.—Tea powder (7.5 g) was boiled for about 10 min with 250 ml H_2O, then filtered. The filtrate (tea decoction) was made alkaline with 10% NaOH and extracted with Et_2O until exhaustion (Extract A). Another 7.5-g tea sample was allowed to stand in boiling H_2O for 3 min. It was then filtered, and the filtrate (tea infusion) was exhaustively extracted with Et_2O (Extract B). The tea marc in either case was separately dried, reduced to a fine powder and then percolated with Et_2O to exhaustion (Extract C). Determination of diphenylamine content in the Et_2O extracts A, B, and C was performed by either of the above-mentioned procedures.

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