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[CONTRIBUTION FROM THE CHEMICAL RESEARCH LABORATORY, THE UPJOHN COMPANY.]

SOME CONSTITUENTS OF THE ROOT OF BRAUNERIA ANGUSTIFOLIA.

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In a report of the Council on Pharmacy and Chemistry[1] of the American Medical Association, the root of *Brauneria angustifolia*, commonly

[1] *J. Am. Med. Assoc.*, **53**, 1836 (1909).

known as Echinacea is stated to be without therapeutic value. Nevertheless, there is a sufficient demand for the root to make it profitable for a number of agriculturists, especially in Kansas and Nebraska, to supply the present requirements of the pharmaceutical market. This demand for the drug consists, not only for use in the several patent medicines enumerated in the above mentioned report, but also for nonofficial unadvertised preparations, such as the common fluid extract.

For this reason it has been considered of interest to subject the root to a chemical study, to learn if the chemical evidence gained would be of any value in forming a more definite conclusion upon its therapeutic properties. No physiologically active substance was isolated, and the results herein reported may, therefore, be considered as partially confirmatory to the report of the Council.

The study of the plant was made as nearly quantitative as possible and we have endeavored to state the weights of the many fractions into which the alcohol soluble constituents were separated. A review of the quantities of the indefinite noncrystalline substances, as compared with the weights of the plant constituents which could be identified, indicates that the former products very greatly preponderate. In view of these facts the chemical evidence cannot be considered conclusive.

In an earlier paper[1] from this laboratory the proximate analysis of this root was reported. It was shown that the air-dried root contained 10.9% moisture and 7.8% ash. Ligroin extracted 0.77%; ether, 1.2% and alcohol 19.7% (of which only 0.14% is inorganic). The residue insoluble in alcohol had the following composition: crude fiber, 24.7%; pentosans, 15.6%; protein, 6.9%; inulin, 5.9%. An "inuloid" product amounting to about 6.0% was also present. These results account for about 98% of the plant. The presence of inulin and inuloids rather than starch and dextrins causes this summation to be subject to some error, because of the lack of well-known methods for the determination of inulins.

The alcoholic extract showed the presence of about 7.0% sucrose and 4.0% l vulose. The resin amounts to 1.9% of the air-dried plant. Of the 19.7% dissolved by alcohol these substances amount to 12.9%, leaving 6.8% unaccounted for.

We report the examination of the products present in the alcoholic solution and soluble in water, and of the ligroin, ether, chloroform, ethyl acetate, and alcoholic extracts of the resin which was insoluble in water.

The aqueous solution yielded traces of a phenolic acid agreeing in composition with the formula $C_9H_{10}O_5$ and melting at 207°; an amorphous glucosidic amyl alcohol extract amounting to 0.3%; betaine, 0.1%; and the sugars above mentioned.

The ligroin extract of the resin yielded oleic, linolic, cerotic and palmitic

[1] *Am. J. Pharm.*, **86**, 451 (1914).

acids; two isomeric phytosterols, $C_{27}H_{46}O$, one melting at 154.5–156.5° and forming an acetate melting at 131.5–132°, while the other phytosterol melted at 136–137° and formed an acetate melting at 118–120°. The ether extract yielded a phytosterolin melting at 280–290° and agreeing in composition with the formula $C_{33}H_{56}O_6$. The acetate of this compound melted at 163–164°.

The chloroform, ethyl acetate, and alcoholic extracts of the resin yielded no definite products.

Experimental.

The material used in this investigation, amounting to 33.3 kg. of the air-dried drug, was exhausted by percolation with 95% alcohol. The percolate, which amounted to 260 liters, was concentrated under diminished pressure to a volume of 11.5 liters. The resin was completely precipitated by mixing this alcoholic solution with about 20 liters of distilled water. By siphoning, most of the aqueous layer was readily separated from the supernatant resin. We thus obtained two fractions, (1) the plant substances soluble in water, (2) the resin, a brownish yellow product, which failed to give any evidence of crystallization from solution in 95% alcohol at various concentrations.

Examination of the Aqueous Solution.—This clear reddish brown solution was concentrated under diminished pressure to a volume of about ten liters and extracted repeatedly with large volumes of ether. The ethereal solution, which contained not more than 5 g. of material, was concentrated to a small volume and fractionally extracted with solutions of hydrochloric acid (10%) water, ammonium carbonate, sodium carbonate, and potassium hydroxide successively. The last two solvents extracted nothing which could be identified.

The hydrochloric acid extracts of the ether extract were dark brown in color, and upon standing gave smeary precipitates. Tests with Mayer's reagent and with iodine in potassium iodide solution gave slight but positive results in the first five acid extracts.

Each of the ammonium carbonate extracts was acidified separately. The first extract gave an oil which, after standing several days, showed the presence of a small quantity of a crystalline body. The subsequent extracts gave similar results, but with the presence of smaller amounts of the smeary material. The acid liquid was in each fraction poured off from this precipitate and the residue, after drying in a vacuum over sulfuric acid, was allowed to stand with a small volume of anhydrous ether, which readily dissolved the smear and left a residue of the crystalline material. These products amounted to less than 0.1 g. As the product was obviously impure and insufficient to work with, the original aqueous extract was concentrated sharply to a volume of 5.5 liters and

again extracted with ether. The ether extracts were worked up as before, but the additional quantity of substance isolated was very slight.

The material separating upon acidification of the ammonium carbonate extracts, was united in one fraction and dissolved in absolute methyl alcohol. It was then esterified with dry hydrochloric acid gas in the usual manner. The solvent was removed and the esters dissolved in ether. The ethereal solution was extracted with water and with ammonium carbonate. Most of the product was extracted with dilute potassium hydroxide solution, thus indicating the presence of a phenolic ester. The alkaline extract was concentrated to a small bulk on the water bath in order to saponify the ester. Upon acidification an acid separated in colorless needles, which, after recrystallization from water, melted at about 207°. Upon elevating the temperature in the capillary tube above the melting point the evolution of a gas ensued. This product gave no color test with a solution of ferric chloride and upon analysis gave results which agree with an acid having the formula $C_9H_{10}O_5$, possibly a trioxyphenylpropionic acid.

0.0564 g. subst. gave 0.1125 g. CO_2 and 0.0262 g. H_2O. Calc. for $C_9H_{10}O_5$: C, 54.5; H, 5.1; found: C, 54.4; H, 5.2.

The aqueous solution which had been completely extracted with ether was now extracted repeatedly with hot amyl alcohol. By carefully concentrating the various amyl alcohol extracts there was obtained several crops of brown, amorphous material which, being similar in appearance, were joined. This product could not be crystallized. An acid hydrolysis yielded nothing crystalline. It yielded, however, a quantity of *d*-phenylglucosazone melting at 202–203°.

The filtrates from which the above mentioned brown amorphous product had separated were studied separately but could not be brought to crystallization. When the amyl alcoholic solution was precipitated with ligroin, the material separated as a smear. By precipitation with ether it was found possible to prepare a light, amorphous product from the latter fractions, whereas the products from the first three of the amyl alcoholic extracts were of a syrupy consistency.

All of this material was brought into one fraction. The weight was approximately 95 g. The amyl alcohol fraction therefore amounts to about 0.31% of the root.

Forty-two grams of this material were boiled for one minute with 10% aqueous potassium hydroxide in the presence of sufficient alcohol to retain it in solution. Nothing of interest was isolated; another portion, amounting to 50 g. and entirely free from material reducing Fehling's solution, was boiled with 21 g. of sulfuric acid for $2^1/_2$ hours in the presence of 500 cc. dilute alcohol. On standing over night a heavy smear separated. The supernatant liquid was poured from the resin and dis-

tilled in a current of steam. Although this acid hydrolysis was accompanied by the evolution of a product having an odor similar to furfuraldehyde, the steam distillate failed to give a positive test for this. The steam distillate contained, floating upon the surface, an appreciable quantity of an oil which was not identified.

The acid liquid which had been distilled with steam was extracted with ether. The ether yielded traces of an acid melting at 203–205° and identical with the one previously described.

The aqueous liquid which had been extracted with ether showed, when quantitatively studied as regards its reducing action upon Fehling's solution, the presence of a sugar amounting to 3.2 g. calculated as dextrose. It yielded *d*-phenylglucosazone melting at 203–204°.

The products extracted with amyl alcohol are, therefore, shown to be partially glucosidic in their nature. They possess, in part at least, the bitter taste of Echinacea root.

The aqueous liquid which had been extracted with ether, and with amyl alcohol, was freed from the latter immiscible solvent by means of a vigorous steam distillation. The total volume at this point was 8.8 liters.

100 cc. gave, with lead subacetate, a precipitate containing 0.0871 g. nitrogen.

100 cc. gave, when distilled with MgO, 0.0129 g. nitrogen as ammonia.

25 cc. gave a quantity of nitrogen equivalent to 0.5004 g. per 100 cc. by the Kjeldahl method.

The distribution of nitrogen is therefore as follows: Total soluble nitrogen = 0.132%; ammonia nitrogen = 0.0034%; lead subacetate precipitable nitrogen = 0.023%; nitrogen precipitated with phosphotungstic acid = 0.032%.

Obviously, a large part of the nitrogenous constituents is not accounted for in the above table. In order to test for acid amides such as asparagine and glutamine, one-fifth of the solution was precipitated with mercuric acetate solution, but only a very slight separation took place. Its subsequent examinations for asparagine, glutamine and allantoine were negative.

The remaining four-fifths was completely precipitated with basic lead acetate, whereupon a heavy precipitate separated. This was removed by filtration and decomposed with hydrogen sulfide in the usual manner. This dark-colored solution gave, with ferric chloride solution, a very slight greenish color. The usual tests for tannin were negative. An effort to prepare copper salts from the solution proved futile and an alkaline hydrolysis yielded no definite products. This fraction contained about 168 g. of amorphous material.

The filtrate from the above precipitation was freed from the excess of lead with hydrogen sulfide and after filtering off the lead sulfide, the filtrate was concentrated to a syrup, which contained 2967 g. of material (dried at 100°). A quantity (535 g.) was precipitated with an excess (180 g.) of phosphotungstic acid in the presence of 5% sulfuric acid. A heavy precipitate resulted. This was removed by filtration, washed

with 5% phosphotungstic acid wash and decomposed by the method of Wechsler.[1] The solution of the basic products was made up to a volume of 2000 cc.

25 cc. distilled with MgO yielded no ammonia.
100 cc. required by Kjeldahl method 54.5 cc. 0.1 *N* acid.

Of the 2000 cc. solution, 600 cc. were concentrated to a syrup at 33°. To this was added 350 cc. of absolute alcohol. A separation of 0.72 g. of amorphous material took place. The filtrate from this material was concentrated to a syrup which weighed about 6 g., and it was now readily soluble in absolute alcohol. To this alcoholic solution an excess of concentrated hydrochloric acid was added, and after standing a short time beautiful crystals of betaine hydrochloride formed. Altogether, 1.45 g. of betaine hydrochloride, equivalent to 0.08% of the plant were obtained. The melting point was 237–238°.

Calc. for $C_5H_{11}O_2N.HCl$: Cl, 23.1%; found: Cl, 22.85%.

It formed the double platinum salt, which melted at 242°.

Calc. for $(C_5H_{11}O_2N.HCl)_2PtCl_4$: Pt, 30.3%; found: Pt, 30.8%.

It is evident that the betaine isolated quantitatively as the hydrochloride fails to account for the total nitrogen in this fraction, for the quantity of nitrogen due to betaine is only 0.44 g., against 1.53 g. known to be present in the 2000 cc. solution. This nitrogenous product is present in the filtrate of the betaine hydrochloride but was not identified.

The study of these nitrogenous constituents was repeated upon a larger quantity, following the comprehensive methods outlined by E. Schulze and E. Winterstein.[2] A slight separation of a purine fraction was obtained but nothing could be isolated from it. The arginine and histidine fraction was absent. The material was precipitated for the second time with phosphotungstic acid, and betaine hydrochloride quantitatively separated as before. The alcoholic, neutral filtrate was precipitated with a saturated alcoholic solution of mercuric chloride, whereupon a thick, black oil separated, but no crystalline mercuric salts could be obtained. The oil, as well as the filtrate from it were treated with hydrogen sulfide, but no crystalline products could be prepared.

The other products of the syrupy liquid, as has been shown in another paper,[3] are levulose and sucrose. This syrup yielded a heavy crystalline deposit of *d*-phenylglucosazone melting with decomposition at 203–205°.

Examination of the Resin.—The resin weighed 628 g. It was extracted in the usual manner with various solvents with the following results: ligroin, 222 g.; ether, 107 g.; chloroform, 180 g.; ethylacetate, 35 g.; alcohol, 70.0 g.

[1] *Z. physiol. Chem.*, **73**, 138 (1911).
[2] *Handbuch der Biochem. Arbeitsmethoden*, Vol. II, p. 518.
[3] *Loc. cit.*

The Ligroin Extract.—For the further examination of this material 157 g. were dissolved in 500 cc. of ether. The ether solution was extracted with small portions of 5% and 10% hydrochloric acid solutions. These were made alkaline and extracted with chloroform. The chloroform solution gave a slight test for alkaloids with Mayer's reagent, but on evaporation of the solvent a small, oily residue was left from which nothing was obtained. The ethereal solution was then extracted successively with solutions of ammonium carbonate, potassium carbonate, and potassium hydroxide. The potassium carbonate extract yielded, upon acidification, 40 g. of fatty acids. This was completely soluble in ligroin. The ligroin solution was dried over anhydrous sodium sulfate, filtered and the ligroin removed. The residue distilled under diminished pressure, boiled for the most part from 236–276° at 33 mm. The iodine numbers of the fractions varied from 75.8 to 82.8.

The fatty acids were then separated into their solid and liquid components by the usual method. From 23 g. of the mixed fatty acids, 12.9 g. of liquid acids were obtained, which were distilled under diminished pressure.

Fraction.	Pressure. Mm.	Temperature.	Iodine. numbers.
1	17–18	168–200°	...
2	17–18	210–240	119.7
3	17–18	240–273	116.2
4	17–18	273–294	100.6

A combustion was made on Fraction II.

Calc. for $C_{18}H_{34}O_2$: C, 76.59; H, 12.06; for $C_{18}H_{32}O_2$: C, 77.1; H, 11.4; found: C, 77.44; H, 11.64.

The iodine numbers, as well as the results of the combustion, indicate the liquid acids to be a mixture of *oleic* and *linolic* acids with oleic acid predominating.

The potassium hydroxide extractions of the ethereal solution gave only a slight amount of an oily residue, which was examined in conjunction with the fatty acids obtained after the hydrolysis of the glycerides with alcoholic potash.

After the ethereal solution had been extracted with potassium hydroxide the ether was removed and the residue saponified with alcoholic potash. The alcohol was removed and the residue diluted with water. This alkaline solution was then extracted many times with ether.

The aqueous solution of the potassium salts, after the removal of the unsaponifiable matter by means of ether, was acidified and then again extracted with ether. The ethereal solution was washed, dried and the ether removed. On dissolving the residue in a large volume of low boiling petroleum, a small quantity of resinous matter was precipitated. The petroleum was then removed and the residue distilled under diminished

pressure. The weight of distilled acids was 48.5 g. (iodine number = 101.9). These acids were separated into the solid and liquid components by the usual method. Nineteen grams of liquid acid and 24.6 g. of solid fatty acid were obtained. The liquid acids were then distilled again under diminished pressure. Most of the acid came over at about 200° at 11 mm.

Fraction.	Temperature.	Pressure, Mm.	Iodine number.
1.....	...–175°	11	...
2.....	175–215	11	141.4
3.....	215–230	11	147.7
4.....	230–250	11	118.4

To gain further insight into the nature of these acids they were oxidized by the method outlined by Lewkowitsch.[1]

Dioxystearic acid, melting at 129.5–130.5° was isolated. Sativic acid was obtained upon the oxidation of the liquid acids occurring free in the fat; and also from the acids obtained after hydrolysis. The melting point was 155–158°. The melting point after a large number of crystallizations from alcohol was raised to 170.5–172°.

Calc. for $C_{18}H_{36}O_6$: C, 62.1; H, 10.3; found: C, 61.8; H, 10.5.

The Solid Fatty Acids.—The solid fatty acids amounting to 27.2 g. derived from the hydrolysis of the glycerides were united with those that occurred free in the fat and subjected to fractional crystallization from absolute alcohol. The first fraction that separated, when recrystallized from absolute alcohol, weighed 0.7 g. and melted at 77–78°. After one more crystallization from alcohol it melted at 77.5–78.5°. It was identified as cerotic acid.

Calc. for $C_{26}H_{52}O_2$: C, 78.8; H, 13.1; N. V., 141.7; found: C, 78.6; H, 13.1; N. V., 140.4.

Fraction II after several crystallizations from absolute alcohol melted at 53.5 to 55.5°.

Calc. for $C_{16}H_{32}O_2$: C, 75.0; H, 12.5; N. V., 219.1.
For $C_{18}H_{32}O_2$: C, 76.1; H, 12.7; N. V., 197.5.
Found: C, 75.26; H, 12.33; N. V., 202.8.

This is impure palmitic acid.

Fraction III, after several crystallizations from absolute alcohol, melted at 55–57°.

0.4842 g. neutralized 0.973 g. KOH. Neutralization value[2] = 200.4.

Examination of the Unsaponifiable Matter.—The ethereal solution was gradually concentrated but no hydrocarbons separated. Upon the complete removal of the ether a partially crystalline residue amounting

[1] "Chemical Technology and Analysis of Oils, Fats and Waxes," p. 564.

[2] Lewkowitsch, Vol. 1, p. 518. Tortelli and Pergami obtained a value of 202.7 for palmitic acid when titrated in the cold. This was repeated in this laboratory and the value 204.2 was obtained.

to about 48 g. remained. This material was fractionally crystallized from absolute alcohol and from ethyl acetate.

It consisted in part of a crystalline mixture of phytosterols and also of an oily fraction which remains in the mother liquors after the separation of the less soluble phytosterols. A fraction of these melting at 134–135° was analyzed.

Calc. for $C_{27}H_{46}O.H_2O$: H_2O, 4.8; found: H_2O, 4.7.
Calc. for $C_{27}H_{46}O$: C, 83.9; H, 11.9; found: C, 83.8; H, 11.9.

As the melting points of the various fractions were not constant, the entire fraction was acetylated and 13.2 g. of the acetates were obtained. When tested for the presence of stigmasterol by the method of Windaus and Hauth[1] it gave negative results. The remaining 10 g. were fractionally crystallized from ethyl acetate. By this means two fractions were finally separated, the less soluble melting at 131.5–132°, and the other melting at 118–120°. The former separated in plates while the lower melting compound formed needles. The acetate melting at 131.5–132° was analyzed.

Calc. for $C_{29}H_{48}O_2$: C, 81.2; H, 11.3; found: C, 81.0; H, 10.9.

When hydrolyzed it proved to be a mono-acetate. 0.3904 g. yielded acetic acid equivalent to 11.1 cc. 0.1 *N* KOH while the calculated volume required by the formula $C_{27}H_{45}OCOCH_3$ is 9.2 cc.

The regenerated phytosterol crystallized from ethyl acetate formed well defined plates that melted at 154.5–156.5°. It was dried at 110°.

Calc. for $C_{27}H_{46}O$: C, 83.9; H, 11.9; found: C, 83.7; H, 11.7.

0.3446 g. of the anhydrous phytosterol made up to 20 cc. with chloroform showed a rotation of —1.46° in a 2 dcm. tube, whence $[\alpha]_D^{20} = -42.2$.

The more soluble fraction of the acetate when hydrolyzed yielded a phytosterol that melted at 136–137°. It was dried at 110°.

Calc. for $C_{27}H_{46}O$: C, 83.9; H, 11.9; found: C, 83.8; H, 11.8.

In the course of this fractional crystallization, by means of which the above phytosterol acetates were separated, we obtained a large intermediate fraction, which yielded a series of crystallizations wherein the melting point ranged from 121.5–126°. Whether or not this contained a third isomer such as sitosterol was not established. From the fraction of the acetates melting at 126° a further quantity of the phytosterol melting at about 155° could be prepared.

The syrupy mother liquor remaining after these substances had been isolated was freed from alcohol and subjected to a fractional distillation. It passed over between 210° to 310° at 20 mm. Most of the oil distilled at 240–290°. The fractions were thick, sticky, oils which did not solidify upon cooling.

[1] *Ber.*, **39**, 4378 (1906); **40**, 3681 (1907).

The Ether Extract of the Resin.—Upon concentrating the ethereal solution a quantity (0.4 g.) of material separated which gave the color tests characteristic for phytosterolins. It was purified by recrystallization from dilute pyridine and melted and decomposed at 280–290°.

Calc. for $C_{33}H_{56}O_6$: C, 72.3; H, 10.2; found: C, 72.4; H, 10.3.

The phytosterol glucoside was converted into an acetate which melted at 163–164°.

The ethereal solution from which this compound had separated was extracted with solutions of ammonium carbonate, sodium carbonate, and potassium hydroxide. The last named solution dissolved most of the material, but the further examination of both the potassium hydroxide solution and of the ether failed to yield any crystalline substances.

The Chloroform Ethyl Acetate, and Alcoholic Extracts of the Resin, were examined in an exhaustive manner but nothing could be isolated.

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THE QUANTITATIVE DETERMINATION OF THE AMINO ACIDS OF FEEDING-STUFFS BY THE VAN SLYKE METHOD.

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Studies relating to the amino-acid content of isolated proteins are of fundamental importance in revealing the character of the chemical processes involved in nutrition and in determining the relative nutritive value of the different proteins. The qualitative and the quantitative amino-acid content of most, if not all, of the forty or fifty natural proteins that have been isolated has been determined by such methods as are now available for such work. However, the quantitative amino-acid content of the mixed proteins of our feeding-stuffs is still entirely unknown. It is desirable, if possible, to determine the amino-acid values of feeding-stuffs, since practical animal husbandry men must always deal with certain mixtures of proteins and protein derivatives as they are found in the naturally occurring feeds.

It is at present impossible to calculate the amino-acid content of feeding-stuffs from the amino-acid content of the isolated proteins for two reasons: first, all of the proteins of any one of the common feeding-stuffs, except milk, have not as yet been isolated in a pure state; and second, there are as yet no methods available for the quantitative separation and determination of the proteins of feeding-stuffs, although some of the main proteins of a number of the feeding-stuffs have been qualitatively separated.

As T. B. Osborne has clearly pointed out, it is quite important for us to know, finally, if possible, the proportion of each type of protein con-