CONTRIBUTION TO THE KNOWLEDGE OF THE HAEMOLYTIC ACTIVITY OF *HYDROCOTYLE VULGARIS* L

BY CHR. J. K. MINK

From the Pharmaceutical Laboratory, University of Utrecht, Netherlands*

Received February 2, 1956

THE use of *Centella asiatica* (L.) Urban in the treatment of leprosy has been reported, and from plants growing in Madagascar, Bontems[1] isolated the crystalline glycoside, asiaticoside. Its aglycone is the monobasic triterpene asiatic acid, which belongs to the α-amyrin series[2] and is therefore closely related to other triterpenoid saponin aglycones. Despite this close affinity asiaticoside appeared to have no haemolytic properties[3].

The investigations of Lythgoe and Trippet[4] have shown that plants of *C. asiatica* occurring in Ceylon contained a glycoside that was not identical with asiaticoside. The centelloside they isolated from the fresh Ceylonese plant differed from asiaticoside not only in its aglycone moiety, but also in its sugar components.

The investigations carried out by these authors into the exclusively tropical *C. asiatica* prompted research on how far *Hydrocotyle vulgaris*, occurring exclusively in Europe, might possess corresponding glycosides. Both genera belong to the tribe Hydrocotyleae, sub-family Hydrocotyloideae of the family Umbelliferae, and are, therefore, botanically closely related. The aims of the work were to ascertain whether asiaticoside is present or not in *H. vulgaris*, applying the method of Bontems; should a glycoside other than asiaticoside be present, to investigate the most suitable method of extraction, isolation and chromatographical examination together with an estimation of haemolytic activity.

THE EXAMINATION OF *Hydrocotyle vulgaris* FOR ASIATICOSIDE AND FOR HAEMOLYTIC ACTIVITY

Commencing with 1500 g. of the entire fresh plant, including subterranean organs, of *H. vulgaris*, the extraction method of Bontems[1] was followed. About 600 mg. of a white amorphous solid was isolated which on ignition yielded a residue of about 20 per cent.; the isolate was slightly soluble in water, less so in acid and, on shaking, the aqueous solution gave much persistent froth; it possessed haemolytic activity and, after hydrolysis, glucuronic acid was split off. These facts led to the assumption that acid saponins are present. Subsequent investigations yielded no evidence of the presence of asiaticoside in this mixture of saponins.

Throughout the remainder of the work to be described in this paper the whole plant, including subterranean organs, of *H. vulgaris*, dried *in vacuo* over P_2O_5 and reduced to coarse powder (No. 20 U.S.P.), has been used for extractions.

The haemolytic index (HI) of the material was determined by the method of Runge[5], using dilutions in geometric gradation. Digitonin was initially

* Department of Pharmacognosy, Director Professor Dr. O. F. Uffelie.

used as a standard but this was later replaced by a purified saponin preparation from the plant itself. Two extraction methods were used: (i) One g. of powdered drug was mixed with 0·4 g. of washed sand, triturated with ethanol 60 per cent. v/v, transferred to a percolator, covered with more solvent, allowed to stand 15 to 20 hours and then percolated with further solvent until, in about 2 hours, 50 ml. of percolate was obtained. Ten ml. of percolate was evaporated to dryness at 70 to 80° C. (it has been shown that this temperature has no influence on the haemolytic action), the dry residue was taken up in 10 ml. of phosphate buffer pH 7·4, warming to 70° C. if necessary, and centrifuging off any undissolved material. The haemolytic index of this solution was then estimated according to Runge's method. (ii) One g. of powder was continuously extracted for 6 hours with chloroform, the extract being rejected. The powder was then extracted with methanol for 7 to 8 hours and the extract adjusted to 50 ml., 10 ml. of which was then evaporated to dryness at 70 to 80° C. and treated as in (i) above. Both methods of extraction yielded practically equal haemolytic index values, and the second method is regarded as more suitable.

This second method of extraction was applied to samples of senega root and sarsaparilla root as well as to *H. vulgaris*. The haemolytic index values for the three drugs are shown in Table I, and it will be seen that the activity of *H. vulgaris* is greater than that of sarsaparilla, and is approximately equal to that of senega and for this reason a more detailed study of the saponins present in the plant was undertaken.

TABLE I

HAEMOLYTIC INDEX FOR 3 DRUGS

Drug	Haemolytic index
Senega root	1925
Sarsaparilla root	1117
Hydrocotyle vulgaris herb ..	1811

ISOLATION OF SAPONIN FRACTIONS

The isolation of saponins from vegetable material using barium hydroxide, magnesium oxide or lead acetate has been shown to result in considerable losses[6]. The method used by Bontems[1] employs lead subacetate and it was rejected on this account. To effect the isolation, a method of continuous extraction with different organic solvents was investigated using successively light petroleum (40° to 60° C.), ether, chloroform, ethyl acetate and methanol, the introduction of ethyl acetate being based on the observation of Rosenthaler[7] that prosapogenins in aqueous medium are easily extracted by shaking with this solvent.

A preliminary experiment was set up with 1 g. of the drug, and continuous extraction, successively using the different solvents for varying times, was applied. The weight of material extracted by each solvent was determined after drying at 80° C. to constant weight and the whole was taken up in 10 ml. phosphate buffer pH 7·4 (if necessary by heating to 70° C.), after which its haemolytic index was determined according to the

method described above. The index was calculated on the basis of the weight of solid (HI_S) and also in relation to the original weight of drug (HI_{Dr}). The HI_S values give some indication of the purity of the extracted haemolytically active substances, the higher the value the more pure the extract.

The results are shown in Table II where it will be seen that repeated extractions were made by both ethyl acetate and methanol. Extraction with chloroform, light petroleum and ether produced very little or no loss of haemolytic activity and since chloroform gave a better extraction of colouring materials it is preferable as a decolourising solvent. Ethyl acetate appeared to extract one group of haemolytically active constituents during the first two or three hours of continuous extraction; more prolonged extraction yielded only a slow and incomplete further extraction. Methanol rapidly extracted the haemolytic activity remaining after ethyl acetate treatment.

The above investigation shows that powdered *Hydrocotyle vulgaris* may be decolourised by the action of chloroform practically without loss of haemolytic activity; from this material one fraction of saponins may be isolated by continuous extraction with ethyl acetate; subsequent treatment of this partially exhausted powder with methanol yields further saponin fractions. This method of isolation was next applied on a larger scale and the two fractions were examined chromatographically on paper both before and after electrodialysis as follows: 250 g. dry powdered drug was mixed with an equal weight of washed sand, made into a paste with chloroform, divided amongst five Soxhlet extractors (Quickfit EX/83/200) and extracted with chloroform for 20 hours, the extract being rejected. The powdered drug was re-dried and extracted with ethyl acetate for 5 to 6 hours, the ethyl acetate extract being reserved. The drug was re-dried and extracted with methanol for 14 hours, the extract being reserved.

The ethyl acetate extract, on standing, deposited a precipitate on the walls of the flask; the solution was decanted, the solvent evaporated almost to dryness under reduced pressure and the residue dissolved in the minimum quantity of methanol 90–98 per cent. The precipitate in the first flask was dissolved in about 50 ml. of warm methanol 80 per cent. v/v and the two solutions were then combined and evaporated at 60° C. to a volume of 30–40 ml. This was allowed to stand for 24 hours in a closed vessel at room temperature, after which it was decanted from any precipitate which formed. The clear solution thus obtained was slowly diluted with 200 ml. ether with continuous mechanical stirring, was allowed to stand at 0° C. for 24 hours, after which the ether layer was decanted leaving a viscous residue. This saponin residue was diluted to about 20 ml. with water and precipitated by adding 4 ml. of 4N sulphuric acid dropwise. After standing at 0° C. for 24 hours the precipitate was centrifuged and washed twice with a little water. It was again centrifuged and was "impure ethyl acetate saponin"; 40 mg. (corresponding to 20 mg. dry substance) was dissolved in 0·4 ml. of methanol and used for paper chromatographic investigation.

The remainder of the impure ethyl acetate saponin fraction was

TABLE II

RESULTS OF SUBJECTING THE DRUG TO EXTRACTION WITH SOLVENTS

Solvent	No.	Experimental series I				Experimental series II			
		a	b	c	d	a	b	c	d
		Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S	Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S
Light petroleum	1	2	1·68	0	0	2	n.d.*	n.d.	n.d.
Ether	2	—	—	—	—	2	0·91	0	0
Chloroform .. (1st)	3	—	—	—	—	4	3·14	20	637
,, .. (2nd)	4	—	—	—	—	—	—	—	—
Ethyl acetate .. (1st)	5	2	6·16	312	5077	3	8·51	316	3714
,, ,, .. (2nd)	6	—	—	—	—	1	1·61	50	3106
,, ,, .. (3rd)	7	—	—	—	—	1	0·72	35	4862
,, ,, .. (4th)	8	—	—	—	—	—	—	—	—
,, ,, .. (5th)	9	—	—	—	—	—	—	—	—
Methanol .. .. (1st)	10	4	17·79	650	3654	3½	15·12	648	4285
,, ... (2nd)	11	—	—	—	—	1	3·50	149	4257
,, ... (3rd)	12	—	—	—	—	2	1·91	60	3141
,, ... (4th)	13	—	—	—	—	3	1·03	50	4913
,, ... (5th)	14	—	—	—	—	4	0·82	0	0

Solvent	No.	Experimental series III				Experimental series IV			
		a	b	c	d	a	b	c	d
		Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S	Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S
Light petroleum	1	—	—	—	—	—	—	—	—
Ether	2	—	—	—	—	—	—	—	—
Chloroform .. (1st)	3	6	6·06	20	331	6	5·55	20	360
,, .. (2nd)	4	1	0·29	0	0	—	—	—	—
Ethyl acetate .. (1st)	5	6	11·08	453	4090	—	—	—	—
,, ,, .. (2nd)	6	1½	1·56	60	3847	—	—	—	—
,, ,, .. (3rd)	7	2½	1·24	34	2743	—	—	—	—
,, ,, .. (4th)	8	2	1·05	29	2762	—	—	—	—
,, ,, .. (5th)	9	3	1·66	60	3615	—	—	—	—
Methanol .. .. (1st)	10	6	13·68	648	4735	6	31·52	1944	6172
,, ... (2nd)	11	1½	0·60	0	0	2	0·79	29	3671
,, ... (3rd)	12	—	—	—	—	—	—	—	—
,, ... (4th)	13	—	—	—	—	—	—	—	—
,, ... (5th)	14	—	—	—	—	—	—	—	—

* n.d. = not determined.

dissolved as completely as possible with very little 0·5N NaOH until just alkaline to phenolphthalein. This solution was filtered through an IG4-crucible and dialysed by means of electrodialysis, using "Ultra-cellafilter, allerfeinst" membranes, a platinum gauze electrode as anode and a silver gauze electrode as cathode. Electrodialysis was continued until the current at 200 volts amounted to only 6–8 milliamp. The contents of the middle chamber were then evaporated in an oven at 70° C. The residue on drying was dissolved in 5 ml. methanol; after storing at 0° C. for 24 hours it was filtered through an IG4-crucible and, after washing the filter with 2–3 ml. methanol, the filtered solution was slowly diluted under mechanical stirring with 120 ml. ether, when the saponins were precipitated. After storing for 20 hours the precipitate was filtered off, washed with ether and dried at 70° C. for 25 minutes. This dried precipitate, yield 250–300 mg., is the saponin fraction provisionally indicated as "dialysed ethyl acetate saponin." Twenty mg. of this fraction

was dissolved in 0·4 ml. methanol and used for paper chromatographic examination.

The methanol extract may contain precipitated material, but this can be rejected if the volume is more than 175 ml. in each flask. The filtered methanol extract was evaporated under reduced pressure (15 mm. Hg.) to 80 ml. which was then slowly diluted with 650 ml. ether with mechanical stirring, and was stored for 24 hours at 0° C. The viscous residue left after decanting the ether layer was diluted to 300–350 ml. with water at 60° C. After cooling, 25 ml. of 4N sulphuric acid was added dropwise with mechanical stirring. On standing at 0° C. for at least 24 hours the saponin precipitated was separated by centrifuging and was washed twice with 150 ml. of 1 per cent. sulphuric acid. About 40 mg. (corresponding to 20 mg. dry substance) of this "impure methanol saponin" was dissolved in 0·4 ml. methanol and used for paper chromatographic investigation.

The remainder of the impure methanol saponin was dissolved in a small quantity of N or 2N caustic soda until the resulting solution was just alkaline to phenolphthalein. Undissolved residue was separated by centrifuging and the clear supernatant liquid was again acidified with 4N sulphuric acid to Congo red, and the above treatment was repeated. This alkaline solution was filtered through a IG4-crucible and the filtrate, diluted with water to 70 ml., was subjected to electrodialysis until the current at 200 volts amounted finally to only 8–10 milliamp. The contents of the middle chamber were evaporated at 70° C. and the residue on drying dissolved in 30 ml. methanol, stored at 0° C., filtered and diluted with 300 ml. ether with mechanical stirring. After standing for 24 hours the precipitate was collected, washed with ether and dried for 25 minutes at 70° C. The white-yellow powder, "dialysed methanol saponin," was obtained in a yield of 3–3·5 g. A solution of 20 mg. of this dialysed methanol saponin in 0·4 ml. methanol was used for paper chromatographic investigation.

The Paper Chromatographic Investigation of Saponin Fractions

Heftmann and Heyden[8] have demonstrated that steroid sapogenins and their acetates were specifically detectable after paper chromatography by spraying the chromatogram with a 2–10 per cent. suspension of rat or guinea-pig blood cells. In 1954 Fiedler[9] made use of citrated ox blood which had been diluted with physiological sodium chloride solution in the ratio 1 : 8.

Method. Whatman No. 1 filter paper was used, spots containing 75–450 μg. being applied at intervals of 2·5 cm. The ascending method of paper chromatography according to Williams and Kirby[10] was employed, the development taking place at 20° C. in an airtight container. When the solvent had travelled about 28 cm. the chromatogram was removed and the solvent front marked.

After investigating the solvents applied by Fiedler, some introduced by Partridge[11] and Jermyn and Isherwood[12] for the paper chromatography of

sugars were found to give a maximal separation of the saponin fractions. These were:

(*p*) benzene/*n*-butanol/pyridine/water..	-1:5:3:3 (upper layer)
(*q*) ethyl acetate/pyridine/water ..	-2:1:2 („ „)
(*r*) phenol/ammonia solution 10 per cent./water (trace HCN).. ..	-75:10:15 (homogeneous layer)

Solvent *p* appeared to be suitable for routine investigation, while solvent *r* gave fine chromatograms but was not suitable for the examination of the "impure ethyl acetate saponin," as two components (Component-S_4 and Hydrocotyle-saponin A_2, see below) could not be separated.

The chromatograms obtained with the solvents *p* and *q* were dried for 40 minutes at 70–80° C. and then sprayed with citrated ox blood diluted with the phosphate buffer pH 7·4 according to Runge in the ratio 1:7. The chromatograms obtained with solvent *r* were first dried at 70° C. for one hour; after that they were exposed to the air for 20 hours before spraying.

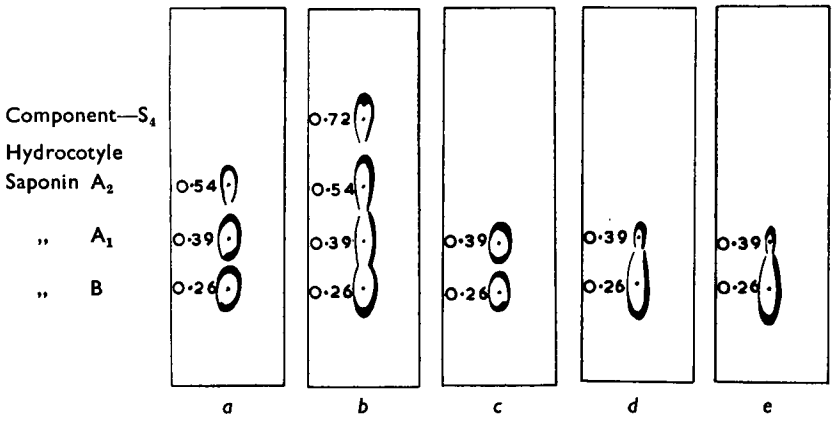

FIG. 1. Chromatograms obtained, using solvent *p*, with the ethyl acetate and methanol fractions of *H. vulgaris*.

a. Impure ethyl acetate saponin.
b. Impure ethyl acetate saponin (high concentration).
c. Dialysed ethyl acetate saponin.
d. Impure methanol saponin.
e. Dialysed methanol saponin.

The chromatograms obtained using solvent *p* with the ethyl acetate and methanol fractions of *H. vulgaris* are shown in Figure 1, *a–e*. It was found that the impure ethyl acetate saponin normally shows 3 haemolytically active substances, which are believed to be saponins. These were therefore provisionally called as follows:

with $R_F = 0{\cdot}54$, Hydrocotyle-saponin A_2
with $R_F = 0{\cdot}39$, „ „ A_1
with $R_F = 0{\cdot}26$, „ „ B

If high concentrations were used it was sometimes possible to distinguish a fourth substance with $R_F = 0{\cdot}72$, which was provisionally called Component-S_4. After spraying with the reagent, the spots of Hydrocotyle-saponin A_2 and Component-S_4 appeared first.

After electrodialysis of the impure ethyl acetate saponin, only Hydrocotyle-saponins A_1 and B were observed. Hydrocotyle-saponin A_2 and Component-S_4 passed through the membrane but it seems probable that

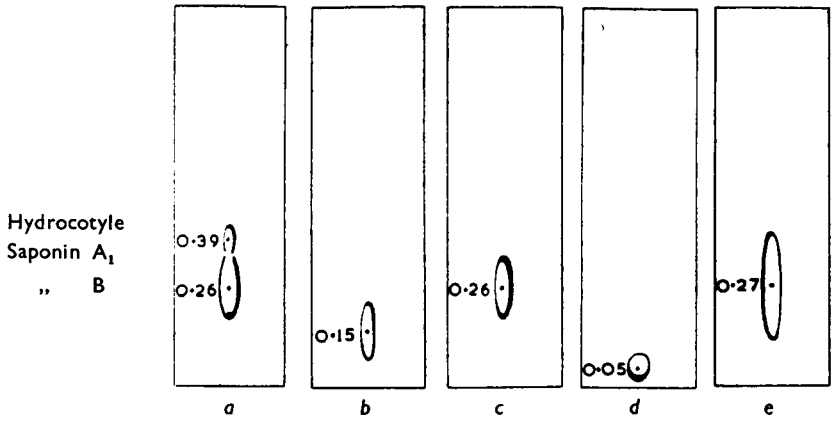

FIG. 2. Chromatographical comparison, using solvent *p*, of:
a. Methanol total extract of *H. vulgaris*.
b. Methanol total extract of senega root.
c. Saponin fraction obtained according to Bontems' isolation method.
d. Quillaia saponin.
e. Digitonin.

they are saponins, since the aglycone of Hydrocotyle-saponin B and of some commercial saponins were found again in the solvent front of the solvent *p*; they may be crystalline saponins, which pass through the membranes more quickly than amorphous saponins.

Both the impure and the dialysed methanol saponin fractions behaved in the same manner and appeared to consist of Hydrocotyle-saponin B with a small amount of Hydrocotyle-saponin A_1. The comparatively large yield (3–3·5 g.) of dialysed methanol saponin means that from a quantitative point of view Hydrocotyle-saponin B forms the most important haemolytically active component of *H. vulgaris*.

Using solvent *p*, the haemolytically active substances of *H. vulgaris*, obtained by continuous extraction with methanol of 1 g. of drug for 8 hours after a 6-hour extraction with chloroform, were compared chromatographically with (i), a methanol extract of a senega root prepared as for *H. vulgaris*; (ii) a solution in methanol of the saponin preparation obtained according to the isolation method of Bontems (50 mg./ml.); (iii) a solution of quillaia-saponin in water (50 mg./ml.); (iv) a solution of digitonin in methanol (50 mg./ml.). These chromatograms are shown in Figure 2, *a–e*, from which it will be seen that the methanol total extract of *H. vulgaris* gave, in high concentrations, only 2 components, namely:

Hydrocotyle-saponin A_1 and B with the regular R_F values 0·39 and 0·26. The methanol total extract of senega root yielded one spot, senegin, with $R_F = 0{\cdot}15$. The saponin preparation obtained according to Bontems' isolation method showed one spot with R_F value = 0·26 which appeared to be identical with Hydrocotyle-saponin B. The quillaia-saponin appeared as a fine, nearly round spot and had a R_F value = 0·05. Digitonin in low concentrations gave less fine, elongated spots with R_F values that ranged between 0·25 and 0·30. It thus appears that other saponins may be distinguished from those occurring in *H. vulgaris* when examined by paper chromatography using solvent *p*.

Summary

1. A systematic investigation of *Hydrocotyle vulgaris* using small quantities of the dried herb has shown the presence of two different saponin fractions, which were isolated by continuous extraction with ethyl acetate and with methanol.

2. Using paper chromatography four haemolytically active substances can be detected. A dilution of citrated ox blood with the phosphate buffer pH 7·4 according to Runge in the ratio 1 : 7 serves as a specific detection of the saponins on the paper.

3. The herb possesses haemolytic activity equal to that of senega root.

4. Two methods of extraction are given for determining the haemolytic index of the dried herb.

References

1. Bontems, *Bull. sci. pharmacol.*, 1942, **49**, 186.
2. Polonsky, thesis, Paris, 1953.
3. Grimes and Boiteau, *8th Rapp. Ann. Parc. Bot. et Zool.*, Tananarive, 1947.
4. Lythgoe and Trippett, *Nature, Lond.*, 1949, **163**, 259.
5. Runge, *Pharm. acta Helvet.*, 1952, **27**, 315.
6. Kofler, *Pharm. Mh.*, 1922, p. 117.
7. Rosenthaler, *Ztschr. Unters. d. Nahrungs und Genuszm.*, 1913, **25**, 154.
8. Heftmann and Heyden, *J. biol. Chem.*, 1952, **197**, 47.
9. Fiedler, *Arzneimitt.-Forsch.*, 1954, **4**, 213.
10. Williams and Kirby, *Science*, 1948, **107**, 481.
11. Partridge, *Biochem. J.*, 1948, **42**, 238.
12. Jermyn and Isherwood, *Biochem. J.*, 1949, **44**, 402.