THE CHEMICAL ASSAY OF CASCARA

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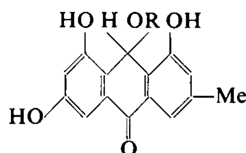
The bark of cascara (*Rhamnus purshianus* DC.) is believed to contain free emodin, aloe-emodin, and chrysophanol. The purgative activity of the drug is thought to be due not to these free anthraquinones, but to glycosides of the anthranols (or of the oxanthrones) corresponding to some or all of them. Little is known about the structure of such glycosides, except that Schindler[1] has reported the isolation of a glycoside of emodin oxanthrone (I).



(I) R = glucose

Several methods for the assay of cascara have been published. Some of these attempt the determination of total anthraquinones, usually by colorimetric measurement of the red colour produced by the action of alkali on anthraquinones (Bornträger reaction). The results so obtained cannot be expected to measure the therapeutic activity of the drug if, in fact, this activity resides mainly or entirely in the glycoside derivatives.

Kussmaul and Becker[2] described a method for the assay of the sennosides (the glycosides isolated from senna by Stoll, Kussmaul and Becker[3]). They also applied the method to the assay of glycosides in the crude drug, and in galenicals prepared from it. Fairbairn[4] and others have developed this method, and applied it to senna[5–8], rhubarb[9], and cascara[10]. Fairbairn and others also used, for comparison, a biological assay devised by Lou[11].

There is certainly a need for a reliable assay of cascara preparations. The British Pharmacopœia standards for the dry extract (the preparation probably used in greatest amount) are of little value in determining its quality and therapeutic value. In fact, there are extracts on the market that answer the B.P. requirements, yet are sold at a price that would scarcely cover the cost of the cascara bark required to make a B.P. extract. Such products are evidently suspect. One such extract was kindly tested for us by Dr. Fairbairn, using Lou's method. It was found to have an extremely low purgative action, while results obtained by Fairbairn and Mahran's[10] chemical assay method were also unusually low. It is to be expected that the cheapest cascara extracts that meet the requirements of the British Pharmacopœia will often be used. If such extracts, and in particular, tablets made from them, are of low therapeutic value, it is

obvious that cascara may become unjustifiably discredited. It was these considerations that prompted us to commence the work, the first part of which is described in the present paper.

EXPERIMENTAL AND RESULTS

Fairbairn and Mahran's Method[10]

Initially, we applied this method to dry extract of cascara (the same sample was used throughout the work now described). This method comprises the following steps.

1. Chloroformic extraction of free anthraquinones from an aqueous solution of the extract adjusted to pH 3 with hydrochloric acid.
2. Hydrolysis of the glycosides by heating the extracted aqueous solution, adjusted to about 3·3N with sulphuric acid after the addition of hydrogen peroxide, for 15 minutes in a boiling water bath.
3. Ether extraction of liberated aglycones from the acid mixture. The ether solution is successively washed with several portions each of 10 per cent. w/v aqueous sodium metabisulphite and of 1 per cent. w/v aqueous sodium bicarbonate.
4. Extraction of the aglycones from the washed ether solution by shaking with N sodium hydroxide, followed by oxidation with hydrogen peroxide to convert any reduced forms into anthraquinones. The solution is acidified, and extracted with ether. The ether is re-extracted with N sodium hydroxide. From the absorption of the alkaline extract at 500 mμ (photo-electric measurement) the content of aglycones, calculated as aloe-emodin, is determined from calibration curves prepared from tests on pure aloe-emodin. The results are calculated as percentages of aloe-emodin in the sample.

Our results by this method were far from satisfactory, ranging from 0·6 to 1·4 per cent. It was soon realised that variations were mainly determined by the hydrolysis procedure; that the prescribed conditions did not appear to secure complete hydrolysis; and that more prolonged heating sometimes gave higher results. Furthermore, solutions from which the aglycones had been completely extracted were found, after re-heating, *especially* if additional hydrogen peroxide were added, to yield a further quantity of material behaving as aglycone; and still further quantities could be obtained by repetition of this treatment, and without reaching finality. We also found that the colour of the final test solutions faded rapidly on exposure to daylight. Besides avoiding such exposure, we adopted the practice of taking spectrophotometric readings within 30 minutes, whenever possible. Table I gives the ranges of results obtained under various conditions of hydrolysis. The variations were large and unpredictable, and we were unable to find any procedure that gave consistent results.

Hydrogen peroxide was added to the hydrolysis mixture by Fairbairn and Mahran to aid in obtaining the final test solution free from interfering absorption. This was also the purpose of the washing of the ethereal solution with sodium metabisulphite and with sodium bicarbonate. The

TABLE I

COMPARATIVE ASSAY RESULTS OBTAINED ON A SAMPLE OF DRY EXTRACT OF CASCARA, B.P. BY THE FAIRBAIRN AND MAHRAN METHOD[10], AND MODIFICATIONS OF IT

	Hydrolysis procedure	Aloe-emodin* (per cent.)
(*a*)	as described by Fairbairn and Mahran: 15 minutes in boiling water bath ..	0·6 to 1·4
(*b*)	1 hour in boiling water bath	1·0 to 1·6
(*c*)	2 hours in boiling water bath	1·3 to 1·6
(*d*)	boiled under reflux for 15 minutes	0·7 to 1·1
(*e*)	boiled under reflux for 1 hour	0·2 to 0·5
(*f*)	3 periods each of 15 minutes in boiling water bath, with addition of fresh peroxide each time. Aglycones isolated after each treatment.	1st 1·14
		2nd 0·10
		3rd 0·16
	Total ..	1·40

* Our specimen of pure aloe-emodin, in N sodium hydroxide, had E (1 per cent. 1 cm.) = 345 at 500 mμ; this figure was used to calculate these and all subsequent results quoted.

combined effect of these treatments was claimed to eliminate interfering matter, so that the shape of the absorption curve was similar to that of aloe-emodin in alkaline solution. In our hands this was not so. In nearly all instances there was considerable absorption around 400 mμ (Fig. 1), though occasionally, and unaccountably, the form of the curve was much nearer to that of aloe-emodin. If one assumes that the extraneous absorption is caused by material unrelated to the active principles, and this remains to be proved, it can be expected that its presence also causes absorption at 500 mμ, and hence an elevation of the apparent

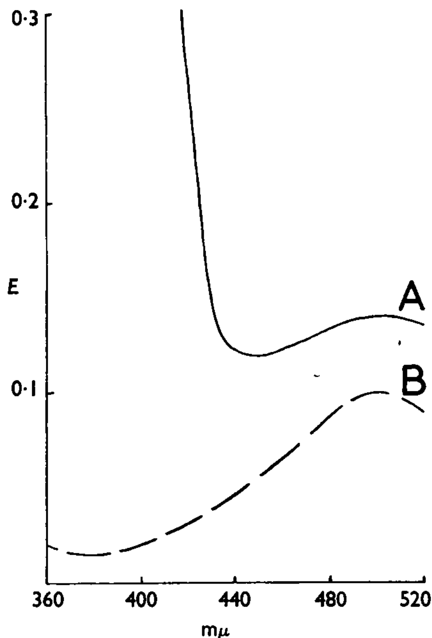


FIG. 1. Absorption curves of (A) cascara aglycones separated by Fairbairn and Mahran's process[10], and (B) aloe-emodin (both in N sodium hydroxide solution).

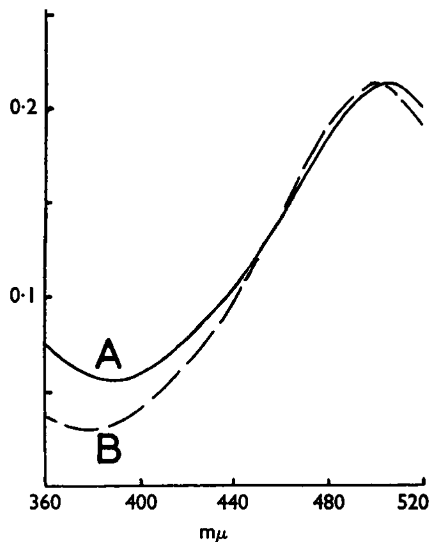


FIG. 2. Absorption curves of (A) light petroleum-extracted cascara aglycones, and (B) aloe-emodin (both in N sodium hydroxide solution).

figures for aloe-emodin. This may account for some of the variation in the above results.

Control experiments with comparable quantities of aloe-emodin, under the prescribed hydrolysis conditions, showed losses of about 20 per cent.; the same applied to alizarin, or to the aglycones isolated from cascara. More prolonged heating increased the loss, which became complete when the mixture was boiled for 30 minutes. Curiously, there is a small loss of aloe-emodin with sulphuric acid alone; but no loss when an equivalent amount of hydrochloric acid (without peroxide) is used instead. These findings caused us to use hydrochloric acid only in our later work.

Because of this action of hydrogen peroxide, it is surprising that any aglycones survived the more prolonged hydrolyses, and we can only speculate about the reason. It is possible that the peroxide is rapidly consumed in attacking other, more readily oxidisable, material. On the other hand, we have found, as mentioned above, that re-heating with fresh peroxide seems to produce more anthraquinone bodies. Whether or not these are derived from therapeutically useful substances remains to be determined. In any event, the results obtained by hydrolysis after the addition of peroxide may well be resultants of simultaneous liberation and partial destruction of anthraquinone derivatives, so that considerable variations could be expected. Accordingly we abandoned the use of peroxide as unreliable.

Similarly, we cannot recommend the use of peroxide in the final (alkaline) stage. Control experiments with aloe-emodin showed a loss of about 10 per cent. under the prescribed conditions, and this was confirmed with algycone solutions prepared from cascara. Furthermore, it appears to us that oxidation is already complete without the addition of peroxide.

Acid Hydrolysis, Benzene Extraction and Chromatography

We then conducted a series of experiments involving hydrolysis of the extract by boiling under reflux for 30 minutes with 3·3N hydrochloric acid, followed by extraction with solvents other than ether. Of those tried, light petroleum (b.pt. 40° to 60° C.) proved highly selective. Direct extraction of it with N sodium hydroxide gave rose-red solutions having spectral curves closely similar to that of pure aloe-emodin (Fig. 2). Unfortunately, the aglycones are so sparingly soluble in light petroleum that it was difficult to secure complete extraction.

Extraction with benzene proved more practicable, but it was considerably less selective than light petroleum, though more so than ether. Partition chromatography on silica gel containing 15 per cent. of water effected considerable improvement, the impurities remaining as a brown band on the upper part of the column.

Further determinations were accordingly made by the following procedure.

Dry cascara extract B.P. (about 40 mg., accurately weighed) was dissolved in water (20 ml.), hydrochloric acid was added to pH 3, and free anthraquinone derivatives were extracted with benzene (5 portions, each of 50 ml.). Each benzene extract was washed with the same portion

(10 ml.) of water. The united benzene solutions were extracted with N sodium hydroxide (4 portions, each of 10 ml.). Residual benzene was removed by subjecting the cold alkaline solution to high vacuum. After suitable dilution, it was examined spectrophotometrically.

The acid aqueous liquid, so extracted, and the water washing were mixed with concentrated hydrochloric acid (15 ml.), and the mixture was boiled under reflux for 30 minutes. The cooled mixture was extracted with benzene (4 portions, each of 25 ml.). The total benzene solution was passed through a 6 cm. × 22 mm. diameter column of silica gel (80/170 mesh) containing 15 per cent. of water. The column was eluted with benzene containing 10 per cent. v/v of chloroform, a bright yellow band gradually passing downwards. Elution was continued until the band was completely removed. The united eluate was extracted with N sodium hydroxide (4 portions, each of 5 ml.). The alkaline solution, after freeing from benzene *in vacuo*, was examined spectrophotometrically in suitable dilution.

The results, and the shape of the curves, for the free anthraquinones were quite constant. The curves showed a peak at 510 mμ, and some interfering absorption (Fig. 3). The relative freedom from interfering absorption suggests that the material causing it is mainly liberated on acid hydrolysis. The results ranged from 0·58 to 0·67 per cent., calculated as aloe-emodin (from the absorption at 510 mμ and using the value E(1 per cent. 1 cm.) = 345).

The results for the aglycones liberated by acid hydrolysis were much less satisfactory. The form of the curves obtained was reasonably good, but with the peak at 505 mμ. (Fig. 3). The results were variable, ranging from 0·3 to 0·6 per cent., calculated as aloe-emodin (from the absorption at 505 mμ, and using the value E(1 per cent. 1 cm.) = 345). More prolonged hydrolysis sometimes gave slightly lower results.

These results were distinctly lower than those we obtained by the Fairbairn and Mahran method. This could be due to elimination of interfering matter that had raised the former results. Again, losses at the chromatographic stage are possible, though control experiments with pure aloe-emodin had given virtually complete recovery. It is also possible that the cascara aglycones are destroyed by boiling with hydrochloric acid, even though aloe-emodin is not. However, the results were still so variable that it was difficult to draw any valid conclusion. Further experiments in which hydrazine hydrochloride was added to the acid hydrolysis mixture, in an attempt to avoid possible oxidative destruction, gave equally unsatisfactory results. Accordingly, we decided that methods involving hot acid hydrolysis were unlikely to yield results of value.

Hydrolysis by boiling for 30 minutes with 10 per cent. w/v aqueous sodium hydroxide was also tried. Much coloured material, but very little aglycone was produced; and subsequent reboiling of the extracted residue, after adjustment to 3·3N with hydrochloric acid, produced only a negligible further amount of aglycone. Thus it appears that alkaline hydrolysis destroys most of the aglycones.

At about this time we obtained an interesting result. The free anthraquinone derivatives had been removed, by chloroform extraction, from a solution of dry extract of cascara, B.P. (1 g.) in water (500 ml.), after adjustment with hydrochloric acid to pH 3. This had been done for convenience, so that aliquot portions could be used directly for hydrolyses under various conditions. The initial results were in good agreement at about 0·62 per cent. of aloe-emodin. Further tests were delayed for 13 days, when the results, again in good agreement, were about 0·78 per cent. This increase of some 25 per cent. surprised us. The solution was

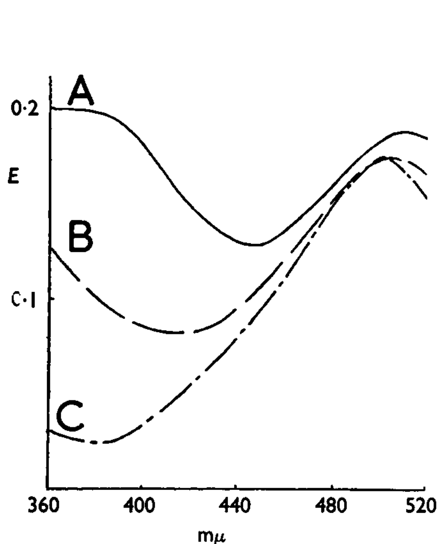


FIG. 3. Absorption curves of (A) free anthraquinone compounds, (B) aglycones (both separated from cascara by the method described in this paper), and (C) aloe-emodin (all in N sodium hydroxide solution).

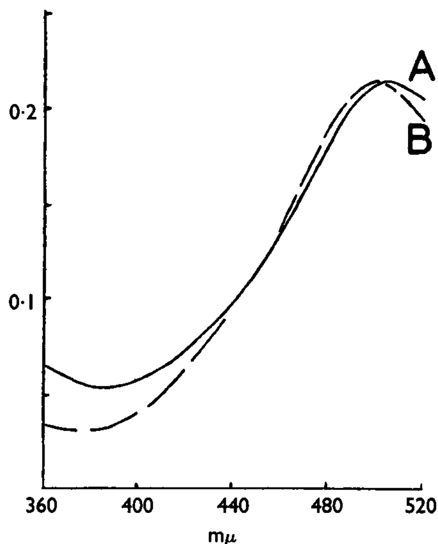


FIG. 4. Absorption curves of (A) cascara aglycones produced by slow hydrolysis in the cold, and (B) aloe-emodin (both in N sodium hydroxide solution).

tested for the presence of aglycones by benzene extraction, and direct extracton of the benzene (not chromatographed) with N sodium hydroxide. A rose-red solution was obtained, despite the initial complete removal of free anthraquinone derivatives from the cascara solution. This indicated that slow hydrolysis had occurred on standing. More important, the spectral curve of the aglycones (Fig. 4) was almost identical with that of pure aloe-emodin, and the absorption at 505 mμ corresponded to 0·37 per cent. of aloe-emodin. After a total of 8 weeks, a further test showed 0·82 per cent. of free aglycones as aloe-emodin, still with no interfering absorption. This result, higher than that initially obtained by hot acid hydrolysis, suggests that such treatment may destroy some of the aglycones. It certainly shows that slow hydrolysis in the cold liberates aglycones without simultaneous production of interfering matter (*cf.* the relative freedom from extraneous absorption of the solution of free anthraquinone derivatives isolated from the extract).

Hydrolysis Under Mild Conditions

Attempts were then made to effect hydrolysis under similar mild conditions, but in a reasonably short time. It was found that the aglycones could be completely extracted by chloroform without pH adjustment. A solution, so freed, was boiled under reflux at the natural pH (about 5·6) for 4 hours. Little or no hydrolysis occurred. This result would imply that hydrolysis of glycosides should not occur during the ordinary processes of manufacture of cascara extracts; though loss of potency may be caused (as suggested by Fairbairn and Mahran's results[10]), due to other changes of unknown nature. In further experiments, adjustment with hydrochloric acid to pH 3·0 preceded refluxing. Even after 4 hours, results of only 0·31 per cent. of aloe-emodin were obtained, while interfering absorption was observed. Standing for 3 days at room temperature in the presence of 3·3N hydrochloric acid gave only 0·37 per cent. of aloe-emodin, and interfering absorption was again observed.

We are forced to conclude that the method of Fairbairn and Mahran cannot be expected to give satisfactory results with cascara. This is disappointing, in view of the need for a reliable assay. Although our results so far have been almost entirely negative, we consider that it may be helpful to other workers to record them. The work described has suggested to us other lines of approach that are now being investigated.

Summary

1. The chemical assay of dry extract of cascara B.P. has been studied.

2. The method of Fairbairn and Mahran[10] has been found to be unreliable. In particular, the addition of hydrogen peroxide in hydrolysis causes partial destruction of anthraquinones, yet it also appears to liberate additional anthraquinone-like material *ad infinitum*.

3. Light petroleum effects selective extraction of the aglycones, but the process is difficult to complete. A method involving acid hydrolysis, benzene extraction, and partition chromatography is described. Though the aglycones were thus obtained in relative purity, the quantitative results were disappointing, and the method cannot be recommended as an assay process.

4. Prolonged hydrolysis in the cold gives aglycones free from interfering matter, but could not be used in a practicable assay method.

We wish to thank Miss H. M. Perry for assistance with some of the analytical work; and Dr. J. W. Fairbairn for the biological test of a commercial sample of dry extract of cascara B.P.

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DISCUSSION

The paper was presented by DR. W. MITCHELL.

The CHAIRMAN expressed the hope that the authors would attempt to ascertain what happened during a standing period of 26 days.

DR. F. HARTLEY (London) said that the standardisation of cascara was a great challenge. DR. Mitchell had shown that the Fairbairn and Mahran method was unreliable but he had not put forward an alternative method. Many people had reached the same position as the authors, but they had not yet thought it appropriate to publish that work. DR. MITCHELL had obtained higher figures by long term hydrolysis but he had not checked to see what he was determining. It was not known whether the physiological potency was due to glycosides or aglycones.

DR. J. W. FAIRBAIRN (London) said that as a result of Dr. Mitchell's criticisms he had tried two other examples using his own method and obtained reasonably consistent results, e.g., 9·63 mg./g. and another worker 9·0 mg./g., for one sample, but he was sorry to have to confess that when he continued the work he found two rather serious defects, especially with B.P. extracts. First of all, the final colour was not always as pure as it should have been. Secondly, the method did not go to completion as one could continue to obtain more aglycones from the extract after hydrolysis was finished. That had led him to investigate the problems carefully, and he had arrived at the conclusion that there were apparently three glycosides present in cascara; based on (1) emodin (2) chrysophanol and (3) aloe-emodin. The emodin glycoside was easily hydrolysed, in fact, very little survived in the extract, and this possibly explained why curve A in Figure 3, had a peak at about 510 mμ. The peak for emodin is 520 mμ whereas for aloe-emodin and chrysophanol it is 500 mμ thus indicating that in the B.P. extract, emodin occurred as aglycone and not as a glycoside The second glycoside based on chrysophanol seemed less easy to split and survived as a glycoside in the extract, while the third glycoside, aloe-emodin, was difficult to hydrolyse and was possibly the same glycoside as was isolated by Lee and Berger. He had a feeling that the interfering substance was attached to that glycoside. By careful preliminary treatment he had been able to induce the separation of this interfering substance and produce a large measure of hydrolysis. Thus with one sample of extract he had obtained 11 mg./g. of impure glycoside by the old method, but by the new method he obtained 29 mg./g. of very pure material. The new method had been tried on several extracts and it appeared as though the order of activity, as compared with the biological activity, was the same as with the old method. By the new method he still found that the bark was much more active than the extract in proportion to the amount of glycoside, and it was tempting to suggest that the

easily split glycoside which disappeared was responsible for the higher activity. He had not found any loss in pure aloe-emodin treated by his method of oxidation in caustic soda, nor did he find the colour faded on standing, except possibly, in sunlight.

DR. D. C. GARRATT (Nottingham) said he had come to the conclusion that the activities and the results of chemical assay of commercial products were so diverse that it was necessary to adopt another line of approach. The difficulty was that if the extract were kept for any length of time, the biological and chemical assays did not agree. It was difficult to see why a purified active principle or a material such as senna powder itself, which was reasonably uniform in activity, could not be used as a standard.

DR. J. W. ROWSON (London) said that the details of the "interesting result" on page 786 of the paper were not clear. Were the "results in good agreement" obtained by the previously described hydrolysis method which had been already rejected as non-reproducible? If the figure of 0·78 per cent. included 0·37 per cent. of free aglycones, was the 0·82 per cent. free aglycones after 8 weeks also the total or were still further quantities combined?

MR. T. D. WHITTET (London) said that he had prepared extracts by four methods, and Dr. Fairbairn had arranged for some preliminary assays. There was some evidence that there was a considerable loss of activity in all.

DR. W. MITCHELL, in reply, said that for simplicity the results reported in the paper were obtained with one particular extract. Many other extracts had been examined, but it would have led to complication to set out a number of results. The title of the paper was "The Chemical Assay of Cascara" and the first object was to try to find an assay which would give reproducible results, the next stage would be to try to relate them with biological findings. At the present stage all that could be indicated was that the methods available were not capable of giving good results. However, further work, which it was hoped to publish in due course, had led to a method which, with the same extract, gave results as high as 60 mg./g., with no trouble due to interfering absorption, and with good replication of results. Furthermore, the chemical figures on the extract were in quite good correlation with those of the sample of bark from which the extract was made. Replying to Dr. Rowson he said that after standing in the cold at pH 3, anthraquinones appeared in material previously freed from aglycones. These anthraquinones could be extracted and submitted to spectrophotometric examination when no interference was detected. Arising from Dr. Fairbairn's remarks, he commented that his own recent work had caused him to doubt whether the active principles were true glycosides.