SURVEY OF ANTHOXANTHINS—PART II

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In the earlier part[1] were described the methods used for the present survey, and the flavonol composition of (1) Moringa flowers, (2) Neem flowers and (3) Indian podophyllum resin. A number of similar sources known to contain flavonols have further been investigated. These are the flowers of (1) Yellow oleander (*Thevetia neriifolia*), (2) Cambodia cotton (*Gossipium hirsutum*), (3) African marigold (*Tagetes erecta*), (4) *Hibiscus vitifolius* (Tamil: Manjal thuthi), (5) *Hibiscus esculentus* (Hindi: Bhindi) and (6) Yellow roses (*a*) Marechel Neil and (*b*) Lady Hellingden (T.). In most cases the results are interesting and new.

1. *Thevetia neriifolia*

It is an evergreen erect shrub belonging to the family Apocynaceæ, and is well known for its poisonous fruits which contain a cardiac glycoside *thevetin*. Other parts of the plant, such as the leaves and the roots are also considered to be poisonous. The flowers are usually bright yellow and fragrant, but occasionally there are varieties which yield apricot coloured flowers.

Desai and Ahmed[2] were the first to examine the dried flowers and they reported the occurrence of two colouring matters considered to be present as their glycosides. They could not isolate the glycosides, but subjected the crude mixture to hydrolysis with 10% sulphuric acid. The fraction of the aglucone mixture which was insoluble in alcohol was an amorphous brown powder; it did not melt below 360°, and failed to give positive colour tests for the flavone group. Its solution in concentrated sulphuric acid gave a dark green fluorescence. The alcohol-soluble component crystallised from this solvent as yellow needles melting at 260°. It answered the special colour reactions for flavonols and its yellow alkaline solution was decolourised by air. Its solution in concentrated sulphuric acid was yellow with an intense green fluorescence and with alcoholic ferric chloride it gave an intense 'black' colouration. It yielded a tetra-acetyl derivative, contained one methoxyl group and on demethylation gave quercetin. It was therefore believed to be a monomethyl ether of quercetin. As it did not correspond with either rhamnetin or isorhamnetin, it was considered to be a new methyl

ether of quercetin, the 4′-methyl ether. No support either by the degradation or by the synthesis of the compound has so far been provided and the recorded results of analysis for C and H do not agree with the requirements of the proposed monomethyl ether. Later Rao[3] examined these flowers collected and dried at Waltair and reported that they gave only a small yield of a flavonol which was identified as kæmpferol.

For the present survey, the yellow flowers have been collected from different parts of India, Delhi (North), Madras (East Coast) and Cochin (West Coast) and the apricot coloured ones from the Delhi area, and each independently examined. Our results differ markedly from those of earlier workers. In all these cases the pigments are present almost completely as glycosides. Circular chromatography of the aglycones shows the presence of two components having Rf values corresponding to quercetin and kæmpferol. Separation of the glycosides by means of neutral and basic lead salts is found to be inconvenient owing to the presence of large quantities of waxy impurities. But on hydrolysis with mineral acid these impurities are converted into an insoluble condition and could be easily removed, and the aglycone mixture obtained free from them. It could then be conveniently separated by the lead salt method. The component obtained from the neutral lead salt has been identified as quercetin and that from the basic lead salt as kæmpferol, using the methods of mixed chromatography and mixed melting point of the free flavonols and also of the acetates. The yield of the flavonol mixture is roughly 0·15% on the fresh Delhi flowers which works up to 0·2% on the air-dried basis. The flowers do not lose much weight by drying. The proportion of quercetin to kæmpferol is approximately 5 : 1.

2. *Gossipium hirsutum*

Viehoever[4] examined the yellow flowers of the American upland cotton, *G. hirsutum* and obtained from them as the major component quercimeritrin and as the minor component isoquercitrin. The Cambodia cotton widely grown in South India under irrigation belongs to the same species *G. hirsutum* and contains flowers which are only of an ivory colour. Neelakantam and Seshadri[5] examined two samples of these flowers, collected and dried in the seasons of the years 1932 and 1933 respectively. The first collection was found to contain mainly quercimeritrin along with a small quantity of quercetin, whereas the second yielded mainly quercetin along with small quantities of quercimeritrin. No isoquercitrin was detected.

For the present survey a sample of sun-dried flowers has been obtained from Coimbatore. It was rather brittle and had undergone discolouration. Chromatographic analysis of the crude aglucone obtained in the usual way

shows that it consists predominantly of quercetin along with a trace of kæmpferol. The latter could not be isolated by fractionation. When the alcoholic extract of the flowers is concentrated the major portion of the quercimeritrin separates out. The remaining portion is fractionated using neutral lead acetate and basic lead acetate. The neutral lead salt is red and on decomposition yields a glycoside which does not give Pew's reaction and which on hydrolysis yields quercetin. This fraction therefore consists mainly of quercimeritrin, though the presence of quercetin is also possible. The basic lead salt which is yellow and very small in quantity yields on decomposition a glycosidic solution which responds to Pew's reaction and therefore seems to contain a 3-glycoside. On hydrolysis, however, the product is found to be quercetin. It should therefore be concluded that this fraction contains a 3-glycoside of quercetin which is possibly isoquercitrin. It is remarkable that the quercetin derivative like isoquercitrin is not precipitated by neutral lead acetate.[6] The explanation may probably be the existence of some sort of loose combination of the hydroxyl groups in the 3′ and 4′ positions with some other component present in the plant. Such combination is known to take place with sodium borate.[7] Thus the results indicate that the flowers of *G. hirsutum* grown in America and in India have more or less the same composition.

Later a small sample of *G. hirsutum* flowers obtained fresh from the Indian Agricultural Research Institute, New Delhi, towards the end of the last season was examined. It gave a high yield of the pigments (4% on air dried basis) but 50% of it consisted of ether extractable portion (quercetin). Thus it is one of the cases where even fresh flowers contain high quantity of free aglucone.

3. *Tagetes erecta*

These flowers (dried) were examined earlier by Rao and Seshadri[8] and found to contain quercetagetin along with its glucoside quercetagitrin. No other flavonol was reported to be present. The flowers in a fresh condition have now again been examined using the method of circular chromatography. The crude aglucone gives only one ring having Rf value 0·36 which is found to be identical with that given by a synthetic sample of quercetagetin. The total absence of any other component particularly quercetin is quite remarkable. In this respect quercetagetin differs from its isomer gossypetin which is invariably accompanied by more or less quantities of quercetin. This indicates that biogenetically quercetin is more closely related to gossypetin than to quercetagetin. The result is in agreement with expectation from the theory of biogenesis of flavonoids discussed in earlier publications.[9]

4. *Hibiscus vitifolius*

This plant grows wild in the south of India and the flowers are found in abundance during the wet parts of the year. They are sulphur yellow in colour with red eye spots at the base of the petals. Earlier investigations of Rao and Seshadri[10] showed that the fresh flowers form a very good source of the rare 8-glucoside of gossypetin, gossypin. Further it is also a very convenient source for the preparation of gossypetin in large quantities. Smaller amounts of quercetin were also found but since this occurred free it was quite easily separated from the water-soluble glucoside gossypin. In order to see if there could be any other flavonol in this interesting source, a small sample of the fresh flowers has again been examined. They are particularly free from waxy matter and mucilages and hence give clear extracts. The crude aglucone mixture gives only two rings in the circular chromatogram. One of these having Rf value 0·31 is quite bright yellow, gradually turning blue and the other having an Rf value 0·62 is only very weak yellow. These agree with the behaviour of synthetic gossypetin (high concentration) and quercetin (low concentration) respectively. The predominantly major flavonoid component of these flowers is therefore gossypetin (glucoside) with quercetin (free) in very minor quantities as reported by earlier workers.[10]

5. *Hibiscus esculentus*

This plant is grown all over India and is much valued for the green fruits which are used as vegetable. The dried flowers were first examined by Seshadri and Viswanadham[11] who reported the isolation of gossypin in a comparatively poor yield. No quercetin was reported. For the present work fresh flowers have been used. There is considerable difficulty in the extraction owing to the presence of large quantities of mucilages and resins, which also render the isolation of gossypin more difficult. But the aglucone mixture can be obtained satisfactorily pure with less difficulty, the crude yield being 0·3%. Circular chromatography indicates the presence of both gossypetin and quercetin, the former being again the major component, but the ring due to quercetin is brighter than in the case of *H. vitifolius*. Fractionation of the mixture using the acetate method indicates the ratio of the two components to be of the order 9: 1. The pigments are present completely as their glycosides.

6. *Yellow Roses*

Roses are widely distributed in the world, but true species can be met with only in the wild roses; horticultural roses are the products of considerable intermingling and hence pure species are difficult to obtain. Many of them are known only by their horticultural names.

The red roses are largely cultivated. The colouring matter of these was first examined by Willstatter and Nolan[12] and later by Robinson and Robinson.[13] The former isolated cyanin for the first time from the flowers, and the structure of this has been established to be 3: 5-dimonoside of cyanidin.[14] The latter in the course of their survey of anthocyanins reported that the orange red Polyantha roses like Gloria Mundi and Prince of Orange contain pelargonidin 3: 5-dimonoside (pelargonin) in a pure condition. They also found that in one plant some of the flowers were pink and some orange, the pink containing cyanin and the orange pelargonin.

Though not so numerous as the red roses, yellow roses are also fairly common. Here again, several wild species are reported to exist in different countries. The horticultural varieties are generally known by certain horticultural names, *viz.*, Marechel Neil, Lady Hellingden, McGredy's Sunset, etc. Very little is known about the nature of the colouring matter of these yellow roses. In a programme of study of the flavonoid composition of these flowers, Marechel Neil from Delhi and Bangalore and Lady Hellingden from Delhi have been chosen first. Marechel Neil is a climber which bears bright yellow flowers whereas Lady Hellingden (T.) is a bush type, bearing apricot coloured flowers and having the odour of newly opened tea chest. Both these have been found to be rich in flavonoid pigments, which could be readily obtained pure, because the flowers are markedly free from wax and resin. Results of paper chromatography indicate that in both cases, the pigment consists predominantly of kæmpferol with traces of quercetin. Further, by simple recrystallisation of the flavonol mixture, kæmpferol could be obtained quite pure. It would therefore appear that these flowers could be used as highly convenient sources for the preparation of this flavonol. The pigments seem to occur almost completely as their glycosides.

EXPERIMENTAL

1. *Thevetia neriifolia*

Preliminary investigations.—20 g. each of the various samples of the yellow flowers collected from Madras, Delhi and Cochin, were extracted with hot alcohol and the extracts tested for pigments of the flavone group. An immediate deep red colour with magnesium and hydrochloric acid was given in all cases, thus indicating the presence of flavonols. When the extracts were treated successively with neutral and basic lead acetate, yellow precipitates were thrown out, indicating that the pigments were present most probably as their 3-glycosides. However, with zinc and hydrochloric acid only a green colour instead of a pink colour (Pew's reaction) was developed. This may be due to the interference of extraneous matter which the flowers

are found to contain in plenty. The difficulty persisted even after the glycosides had been precipitated as their neutral and basic lead salts and recovered by decomposition with hydrogen sulphide. But if the recovered glycosidic solutions were extracted repeatedly with ether, the interfering impurities were removed. Then the neutral lead salt fraction gave an immediate test with Pew's reagent, whereas the basic lead salt fraction gave the same only after standing overnight. This may be due to the low concentration of the second fraction or the absence of a 3-glycoside in it. The latter is more probable. The ether soluble portion gave no test with magnesium and hydrochloric acid, and also with alcoholic ferric chloride and therefore contained no flavonols.

Next, the total extracts were hydrolysed in the usual way using 7% sulphuric acid, and the aglycones analysed by chromatography. The results as given in the following table, indicated that in all the different cases, a mixture of two flavonols corresponding to quercetin and kæmpferol was present. The Rf values given by these, however, were slightly higher than the values previously obtained.[1] Since Rf values are liable to vary with conditions[15] a comparison with synthetic samples of quercetin and kæmpferol was done under the same conditions, and their Rf values found to correspond to those obtained for the flower extracts.

TABLE I

(*Temp.* 34°)

	Rf_1	Rf_2
Yellow flowers from—		
1. Madras ..	0·60	0·82
2. Delhi ..	0·62	0·81
3. Cochin ..	0·62	0·82
4. Apricot coloured flowers from Delhi	0·62	0·80
Synthetic quercetin ..	0·62	..
Synthetic kæmpferol ..	..	0·80

Further detailed characterisation of the flavonol mixture was done using both the yellow and the apricot coloured flowers collected locally in Delhi in a fresh condition.

Isolation and separation of the flavonol mixture.—400 g. of the fresh flowers collected from the University gardens were extracted thrice with

hot alcohol, concentrated to a convenient bulk and hydrolysed using 7% sulphuric acid. In the course of hydrolysis considerable amount of a dark solid separated out and the solution was green. The solid was insoluble in ether and gave no test for flavonoids. It was filtered off and the alcohol from the filtrate removed by evaporation. The aqueous solution was then extracted with ether and the ether distilled off. The flavonol mixture was obtained as a yellow residue and separation to the different components was effected through lead salt precipitation. An alcoholic solution of the flavonol aglycones was treated successively with neutral and basic lead acetate and the precipitated lead salts separately decomposed with hydrogen sulphide in alcoholic suspension. The two fractions thus obtained were diluted with excess of water, all the alcohol removed by evaporation and the cold aqueous solutions extracted with ether. On distilling off the solvent, the ether extract gave in each case an almost pure yellow residue. The yield of the residue obtained from the neutral lead salt fraction (I) was approximately 500 mg. and that from the basic lead salt fraction (II) only about 100 mg. When analysed by chromatography, the first one corresponded to quercetin and the second one to kæmpferol. They also answered all the colour reactions characteristic of quercetin and kæmpferol respectively.

The quercetin fraction (I) was treated with alcohol. A small part of it remained undissolved and this was filtered off. A pale yellow crystalline substance, yield 50 mg., was left behind. It melted at 218–20°, was practically insoluble in alcohol, did not give any ferric chloride colour and dissolved with difficulty in concentrated sulphuric acid giving a deep yellow solution showing a faint greenish fluorescence. The substance was, however, readily soluble in cold benzene. Thus it did not seem to belong to the group of flavonoids.

The alcohol-soluble part was crystallised twice from alcohol, yield 300 mg. The acetate was prepared in the usual way by boiling the flavonol (300 mg.) with acetic anhydride (3 c.c.) and pyridine (6 drops) for two hours. It crystallised from ethyl acetate as colourless needles, m.p. 190–91°. The mixed melting point with an authentic sample of quercetin acetate was not depressed (Found: C, 58·1; H, 4·4; $C_{25}H_{20}O_{12}$ requires C, 58·6; H, 4·2%).

The kæmpferol fraction (II) was crystallised first from alcohol and then from boiling toluene; yellow needles, m.p. 273–74°. The mixed melting point with a synthetic sample of kæmpferol was undepressed.

The apricot coloured flowers also yielded almost the same proportions of quercetin and kæmpferol and both were present in the form of their glycosides.

2. *Gossypium hirsutum*

The sun-dried flowers (360 g.) were extracted repeatedly with hot alcohol and the extract concentrated. A small quantity of it was hydrolysed and the crude aglucone chromatographed. Two rings were obtained, the inner deep yellow one, Rf value 0·58, corresponding to quercetin and the outer pale yellow one, Rf value 0·76, corresponding to a trace of kæmpferol.

The rest of the extract was then treated successively with neutral and basic lead acetate. The neutral lead salt fraction was deep brown red and indicated the presence of quercimeritrin. It was deleaded, the alcoholic solution of the glucoside hydrolysed and the aglucone chromatographed, when a single ring corresponding to quercetin was obtained, Rf value 0·55. The yield (1%) was much less than what had been reported earlier, and this may be due to too much sun-drying of the flowers or the lateness of the season of collection.

The basic lead acetate fraction was yellow and the alcoholic solution obtained after decomposing the lead salt gave a positive test for Pew's reaction, indicating the presence of a 3-glycoside. This was hydrolysed using 7% sulphuric acid, and the aglycone analysed by chromatography. A single yellow zone agreeing with quercetin was obtained, Rf value 0·56. The alcoholic solution of the aglycone gave all the colour reactions characteristic of quercetin and a red lead salt with neutral lead acetate. Crude yield of the aglycone 138 mg. (0·03%). The flowers therefore contain quercetin, which is very largely present as quercimeritrin and in a minor portion as a 3-glycoside, probably isoquercitrin.

3. *Hibiscus vitifolius*

The flowers (250) were collected fresh from Madras and extracted with boiling alcohol. The extract was hydrolysed with 7% sulphuric acid. Addition of acid imparted a violet red colour to the solution due to the presence of anthocyanins, which remained in the aqueous solution after ether extraction of the flavonols. The aglucone obtained by evaporation of the ether extract was subjected to circular chromatography when two rings were obtained, an intense yellow inner ring, gradually turning blue, Rf value 0·31, and a faint yellow outer one, Rf value 0·62, agreeing with those of gossypetin and quercetin. No other rings were formed, indicating the absence of other flavonols. The results agreed with the reports of earlier workers[10]; quercetin was present mostly free since it could be extracted with ether before glycoside hydrolysis with mineral acid.

4. *Hibiscus esculentus*

Seshadri and Viswanadham[11] employed the dried flowers for study We have here used fresh flowers and ordinary alcohol (95%) for extraction.

The fresh flowers (200 g.) collected from Madras were extracted thrice repeatedly with hot alcohol, the combined extracts concentrated and hydrolysed. On addition of acid, the extract turned deep red due to the presence of anthocyanins coming from the purple spots at the base of the petals. After extracting the flavonol with ether, these remained in the aqueous solution colouring it deep red. The ether extract was coloured green, due to presence of chlorophyll and on evaporation yielded a yellow residue mixed with plenty of a green oily matter. This was removed by shaking the alcoholic solution with animal charcoal. The yield of the purified pigment was 0·55 g. from 200 g. of fresh flowers.

The crude aglucone on analysis by chromatography gave two rings, having Rf values agreeing with those of gossypetin (0·30) and quercetin (0·60). The inner ring corresponding to gossypetin assumed a blue colour in course of time and was very intense, whereas the outer one (quercetin) was weak indicating that the former pigment formed the major portion of the flavonol mixture.

For separation of quercetin, the aglucone mixture (0·35 g.) was acetylated using acetic anhydride and pyridine, and the acetate fractionated from dry ethyl acetate. Most of the acetate mixture was found to be insoluble in ethyl acetate in the cold. This was filtered off and the melting point determined. It was 226–28°, agreeing very closely with that of gossypetin acetate. The mixed melting point with an authentic sample of gossypetin hexaacetate was undepressed.

The second fraction from the mother liquor sintered at 182° and melted between 185–210°. Obviously it was a mixture of the acetates of gossypetin and quercetin. The last fraction (5 mg.) when crystallised from a mixture of ethyl acetate-petroleum ether, melted at 191–92° and corresponded to quercetin acetate. The mixed melting point with a genuine sample of quercetin penta acetate was also the same. Some more quercetin acetate could be obtained by fractionating the second crop of crystals (m.p. 185–210°). It was deacetylated and the product analysed by circular chromatography when a prominent yellow ring corresponding to quercetin was obtained. The relative proportions of gossypetin and quercetin in *H. esculentus* were found to be approximately 9 : 1. Ether extraction before glycoside hydrolysis yielded no free flavonol and hence both gossypetin and quercetin should be present as their glycosides.

5. *Marechel Neil and Lady Hellingden (T.)*

The petals of the fresh flowers (5 g. each) were first extracted with alcohol, hydrolysed with mineral acid and the aglycone analysed by chromatography. In all cases, two zones were obtained, a very faint inner one corresponding to a trace of quercetin, and a bright yellow outer one corresponding to kæmpferol. Thus kæmpferol was present as the predominantly major component and the trace of quercetin could not be isolated.

TABLE II

(*Temperature* 37°)

Source	Number of zones	Rf_1	Rf_2
Marechel Neil (Delhi) ..	2	0·55	0·78
Marechel Neil (Bangalore) ..	2	0·55	0·80
Lady Hellingden (Delhi) ..	2	0·55	0·75
Synthetic quercetin ..	..	0·56	..
Synthetic kæmpferol ..	..	..	0·76

For further characterisation of the pigments, in the case of Marechel Neil from Delhi, petals of the fresh winter flowers (10 g.) were extracted repeatedly with boiling alcohol and the clear deep yellow extract treated successively with neutral and basic lead acetates. In the neutral fraction, a bulky yellow precipitate was obtained, but only a small quantity of a yellow precipitate was formed in the basic lead salt fraction. These were separately decomposed and the glycosidic solutions hydrolysed by 7% acid in each case; yield neutral lead salt fraction, 0·15 g. or 1·5% and basic lead salt fraction, 0·05 g. or 0·5%. The crude aglycones were then separately analysed by circular chromatography. The neutral fraction gave two rings, a bright yellow one corresponding to kæmpferol and a very faint yellow one corresponding to traces of quercetin, Rf values 0·78 and 0·55 respectively. The presence of kæmpferol as the major component in this fraction was further confirmed by preparing the acetate, which lost water at 118° and melted at 180–82°. The basic fraction on analysis by chromatography gave only a single bright yellow ring, Rf value 0·78, again agreeing with kæmpferol. Its melting point when alone or mixed with synthetic kæmpferol was the same. Mixed chromatography with a synthetic sample of kæmpferol also did not produce any other ring.

Flowers of Marechel Neil from Bangalore, collected in summer (10 g.) were also examined in the same way as above. Here also, the major part of the kæmpferol was found to be precipitated in the neutral lead salt fraction. No quercetin could be isolated from it. The yield was, however, much less than in the case of the fresh winter flowers of Delhi. Yield, neutral fraction, 90 mg. and basic fraction, 10 mg. The pigment was confirmed to be kæmpferol by means of the acetate.

It is remarkable that the major portion of the kæmpferol was precipitated by neutral lead acetate and not by basic lead acetate. Pure kæmpferol when not mixed with quercetin has been found to be precipitated partly by means of neutral lead acetate, but the major portion is precipitated by basic lead acetate. The explanation for this extraordinary behaviour of kæmpferol is not clear and is under investigation. It is possible that this is due to the presence of large quantities of basic inorganic materials in these flowers.

In the case of Lady Hellingden, the petals of the fresh flowers (20 g.) were extracted with alcohol, the extract hydrolysed with acid and the aglycone fractionated through the acetate. All the fractions obtained agreed with kæmpferol acetate in their melting point and no quercetin acetate could be isolated. The yield of kæmpferol in these flowers was exceptionally high 2·5% on the wet flowers which works up to almost 10% on the air-dried matter. The flowers lose weight heavily on drying.

SUMMARY

Fresh flowers are found to be much more satisfactory for getting a true picture of the flavonoid composition than dried ones. The following fresh flowers have now been examined. (1) *T. neriifolia.*—The flowers contain a mixture of quercetin (5 parts) and kæmpferol (1 part) present mainly as their glycosides. (2) *Cambodia cotton flowers.*—These contain almost entirely quercetin most of which is present as quercimeritrin and a very small amount is possibly as isoquercitrin. (3) *T. erecta.*—This contains only quercetagetin in the form of its glycoside. (4) *H. vitifolius.*—These contain mainly gossypetin as the 8-glucoside, gossypin, along with free quercetin in minor quantities. (5) *H. esculentus.*—This is similar to *H. vitifolius* except for the presence of large amount of impurities and seems to contain more of quercetin in the form of glycoside. (6) *Yellow roses*: (*a*) *Marechel Neil and* (*b*) *Lady Hellingden* (*T.*) are good sources of kæmpferol and contain quercetin as a very minor component. These are present as their glycosides.

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