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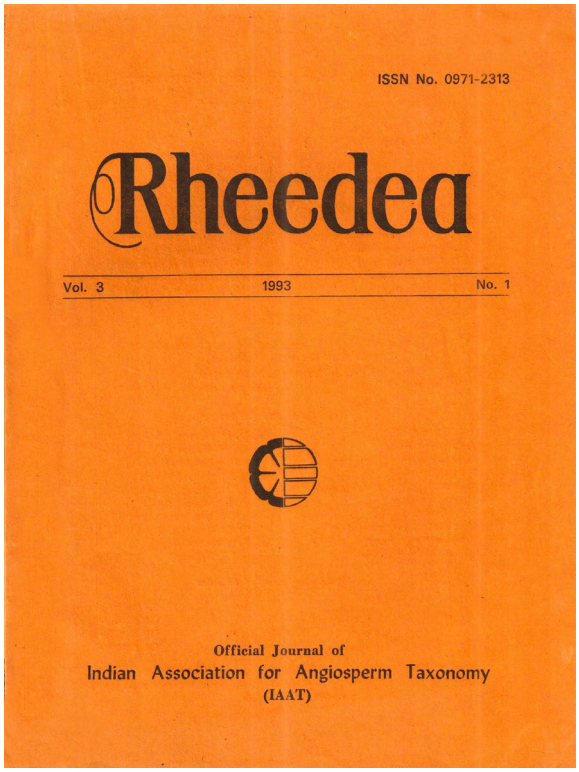

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Flavonoids as chemotaxonomic markers - A review

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Abstract

Among secondary metabolites of plant origin, the flavonoids are the most preferred as chemotaxonomic markers. This brief review highlights the use of these compounds in taxonomic and phylogenetic studies.

INTRODUCTION

Thanks to the pioneering studies of Alston, Bate-Smith, Harborne, Hegnauer, Mabry, Swain and others, chemotaxonomy of higher plants now forms an important branch of phytochemistry and plant sciences (see Hegnauer, 1964 and Swain, 1963 for earlier authoritative and comprehensive reviews). Elaborate classification of plants are made with the ultimate objective of finding out their evolutionary relationships. In the classical approach the identification and classification of plants are made largely on the basis of morphological and anatomical features of the different plant parts (Cronquist, 1968). Classifications based on such methods are not always unambiguous and in several cases additional inputs are necessary to clear doubts. In such cases, comparative phytochemical studies which reveal secondary metabolite profiles often provide additional and independent information of considerable diagnostic value.

Different opinions have been expressed by different authorities regarding the suitability of the term 'Chemotaxonomy'. Erdtman (1968) preferred 'Molecular taxonomy', while Alston and Turner (1963) and Heywood (1973) indicated their preference for the term 'Biochemical systematics'. However, as Erdtman (1968) pointed out it matters little which term is used as long as there is agreement on what it means!

The idea that the chemical profile of a plant can be used for taxonomic purposes is not new. However, the idea was not tested experimentally till 1962, largely due to the lack of comprehensive chemical information. The advent of sensitive and reliable chromatographic techniques made it possible to acquire such information with considerable ease. One of the first to take advantage of this technique was Bate-Smith who applied it to flavonoids. Thus, as early as 1949, he was able to show that the flavonoid composition of Dahlia flowers was much more complex than what it was supposed to be and this resulted in the

availability of a wealth of data till then not known. The impact of this development on Chemotaxonomy was immediate and significant (see, for example, Swain, 1985). Since then, several other advances in experimental strategies have taken place and newer and more sensitive chromatographic methods are now available (Hostettmann. 1985). It should be mentioned here that even before these remarkable developments in phytochemical methodology had occurred, Asahina and Shibata, (1954) had shown the unique value of chemical data in the classification of lichens.

THE ROLE OF SECONDARY METABOLITES IN PLANTS

Naturally occurring organic compounds are broadly classified into primary and secondary metabolites. The former which include amino acids, proteins, carbohydrates, fatty acids and the nucleic acids are essential for any form of life, primitive or advanced. On the other hand, till recently there were differences of opinion with regard to the functions of secondary metabolites, such as the alkaloids, the terpenoids, the flavonoids etc., in plants. It was obvious that life, in its most primitive form, could go on without these compounds but neither could the view that these were waste products of metabolism be accepted (Harborne, 1968). In recent years, a considerable amount of data has become available which clearly show that these compounds do have important functions in plant physiology. For example, several polyphenols and sesquiterpene lactones are known to serve as allelopathic agents (Whittaker and Feeney, 1971). Several instances of secondary metabolite-mediated plant-plant interactions as well as plant-insect and plant-vertebrate interactions are now well-established (Cutler, 1988; Hostettmann and Lea. 1987).

The function of flavonoids in higher plants has been the subject of many studies (McClure, 1975; Stafford, 1990). Earlier studies had shown that flavonoids carrying the catechol oxygenation pattern in the 'B' ring are attacked by the enzyme indole acetic acid oxidase. This, in effect, prevents the enzymatic breakdown of IAA, which is a potent plant-growth hormone. This observation, thus suggested that such flavonoids function in the plants as anti-oxidants preventing oxidative destruction of essential metabolites (Galston, 1969). These observations have subsequently been confirmed and it is now generally accepted that a major function of flavonoids is to act as inhibitors of electron transport in mitochondria. Other possible functions attributed to flavonoids are: (1) as protective agents against ultraviolet light, (2) providing protection against herbivores and pathogens, and (3) as semiochemicals, particularly for attracting bees and insects for help in pollination and seed dispersal (Harborne, 1982).

SECONDARY METABOLITES AS TAXONOMIC MARKERS

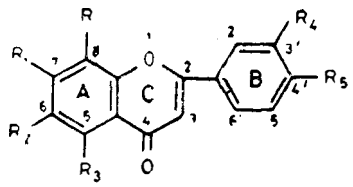
Because of easier expirimental manipulation, secondary metabolites are preferred to primary metabolites for use as markers in taxonomic studies. However,

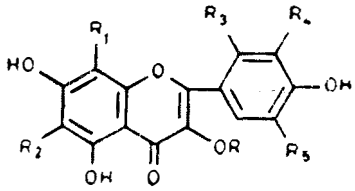
it should be undertstood that, at a future date, when appropriate and adequate experimental techniques become available, the logical choice would be functional primary metabolites such as the enzymes and the nucleic acids. Among the secondary metabolites the choice of any one chemical type for utilization in the classification of plants depends on several factors, both objective and subjective The main objective criterion is the requirement that compounds of the type chosen as markers should occur in a large cross-section of the genus, sub-tribe, tribe or family under investigation. The compounds, while belonging to the same chemical type, such as, for example, indole alkaloids, diterpenes or flavonoids, should exhibit sufficient variation in structural details coincident with variations in morphology and other classical taxonomic features so that meaningful correlations between the two sets of data, namely chemical and botanical, may emerge. For instance, pyrrolizidine alkaloids can serve as convenient and reliable markers in taxonomic studies on plants of the Senecioideae sub-family of the Asteraceae and the *Crotalaria* genus of the Leguminosae. On the other hand, flavonoids and anthocyanin pigments are eminently suited for similar studies on plants of the Malvaceae. The chief subjective factor in the choice of a chemotaxonomic marker is the investigator's speciality; his or her expert knowledge of the chemistry of a particular group of compounds could be a decisive factor since this would ensure the reliability of the experimental observations. Another factor is the ease with which structural variations in a group of secondary metabolites can be unambiguously identified without resorting to highly sophisticated and time-consuming techniques. From this point of view, the flavonoids are, perhaps, the most sought after as chemotaxonomic markers as they can be detected and characterised with the help of relatively simple experimental methods. In this review, we shall touch upon both theoretical and practical aspects concerned with the role of flavonoids in chemotaxonomic studies. The review is restricted to flavones, flavonols and related compounds having the 2-phenylchromone skeletal structure and no reference will be made to the iso- and neo-flavonoids. Regarding practical aspects, the emphasis will be on methods which can be used by taxonomists who may not have the facilities or expertise for isolating pure compounds and to characterise them rigorously by chemical and spectroscopic methods.

STRUCTURAL VARIATIONS AMONG FLAVONOIDS

Flavonoids are derivatives of 2-phenylchromone. Flavone itself, that is, 2-phenylchromone (1). occurs as a dust on the stalks and leaves of plants of the genus *Primula*. It has also been recently detected in two species of *Pimelia* (Thymeliaceae) (Freeman *et al.*, 1981). More common are hydroxylated derivatives. The oxygenation pattern in the two rings marked 'A' and 'B' in the formula shown in chart-1, differ due to different biosynthetic origins of he two parts; the 'A' ring arises by the acetate-malonyl pathway whereas ring 'B' is derived from shikimic acid via phenylalanine and cinnamic acid (Heller and Forkman, 1988). Thus, the predominant oxygenation pattern of ring 'A', as a consequence of its

CHART - 1





1 R = R_1 = R_2 = R_3 = R_4 = R_5 = H

2 R = R_1 = R_2 = R_4 = R_5 = H, R_3 = OH

5 R = H, R_1 = OMe, R_2 = R_3 = R_4 = R_5 = OH

6 R = Gl-Rha R_1 = R_2 = R_3 = R_4 = H R_5 = OH

7 R = $C_6H_{11}O_5$, R_1 = R_3 = R_5 = OH R_2 = R_4 = H

11 R = R_1 = R_2 = R_3 = R_4 = R_5 = H

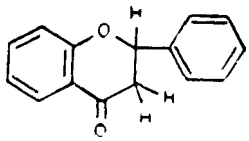
12 R = R_2 = H, R_1 = R_3 = R_4 = R_5 = OH

13 R = R_1 = R_2 = R_3 = R_4 = H; R_5 = OH

14 R = R_1 = R_3 = R_4 = H; R_2 = R_5 = OH

15 R = R_2 = R_3 = R_4 = H; R_1 = R_5 = OH

16 R = R_1 = R_2 = R_3 = H, R_4 = R_5 = OH



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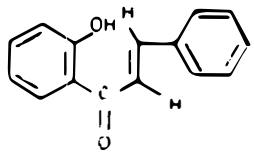

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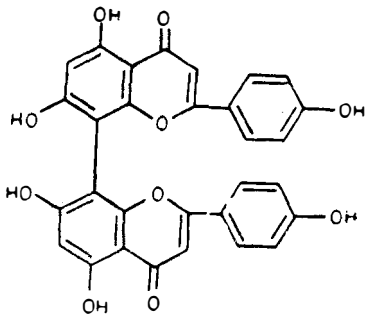

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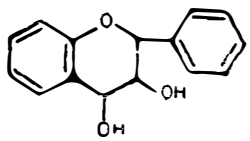
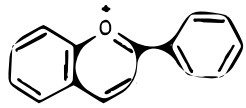


9 (MONOMER)



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byosynthetic origin, is meta-hydroxylation (resorcinol or pholoroglucinol patterns). In 'B' ring, if a single hydroxyl is present, it occupies the 4' position and additional oxygenation is predominantly vicinal in pattern. However, there are exceptions as, for example, morin (2), which is 3, 5, 7, 2', 4'-pentahydroxyflavone. A flavone has a double bond but no hydroxyl in the middle ('C') ring. Flavonol

is 3-hydroxyflavone. A flavanone (3) is dihydroflavone, with the double bond in ring 'C' having been reduced. Flavanones are readily interconvertible with the corresponding 2'-hydroxychalcones (4) in which the middle ring is opened up; both flavanones and chalcones often occur together. The hydroxyls can be further modified either by O-methylation, as in pedalitin (5) or by combination with one or more monosaccharide units to form O-glycosides; a well-known example of the latter type is rutin (6). C-Glycosides, such as vitexin (7), possess a sugar unit which is directly attached to a ring carbon atom. Cupressuflavone (8) is an example of a biflavone in which two flavone moieties are linked together by a C-C bond. Flavone C-glycosides and biflavones are much less widely occurring than the simpler flavones and flavonols. Reductive modification of the middle ring leads to proanthocyanidins (9) and then on to anthocyanins (10). These different types of flavonoids are shown in Chart-1.

METHODS OF DETECTION - DIAGNOSTIC COLOUR TESTS

While the anthocyanins can be readily recognised because of their prominent and characteristic visible colours, the presence of proanthocyanidins, flavones and flavonols in solutions can also be easily detected by means of certain colour tests. Proanthocyanidins get converted into the corresponding anthocyanidins on treatment with warm hydrochloric acid, thus resulting in colour formation. Flavones and flavonols answer the Shinoda test in which an alcoholic solution of the compounds is treated with magnesium powder and concentrated hydrochloric acid (Geissman and Clinton, 1946). The development of an orange or red or a magenta and occassionally a purple colour indicates the presence of a flavone, flavanone or flavonol. This test-tube reaction is sensitive enough to be used for the detection of small quantities of flavones and flavanones provided proanthocyanidins are absent in the extract under examination. The colour formed depends on the degree and pattern of hydroxylation; methyl ethers give less prominent colours. Similar colours are also obtained when an alcoholic soloution of a flavone is treated with sodium amalgam, followed after a few minutes, by hydrochloric acid. Flavonols, their 3-0-glycosides and dihydroflavonols give, in ethanolic solutions, a colour similar to that obtained in the Shinoda test, on treatment with zinc and hydrochloric acid. Most flavones and flavonols dissolve in concentrated sulphuric acid to give yellow-brown solutions which exhibit a bright green fluorescence. Exceptions are 2'-hydroxylated flavones and flavonols which do not exhibit the fluorescence (Ahluwalia *et al.*, 1958). Flavones with a 5, 6, 7-trihydroxy substitution pattern in ring 'A' give a dark green colour, with the separation of a green flocculent precipitate, on treatment with sodium amalgam in ethanolic solution. This test, known as the Bargellini test (Bargellini and Marrini-Bettolo, 1962) has considerable diagnostic value in chemotaxonomic studies. Flavones and flavonols possessing a 5, 8-dihydroxy or 5, 7, 8-trihydroxy pattern answer the 'gossypetone' test in which a solution of the compound in ethanol is treated with ethanolic p-benzoquinone. The development of a golden-yellow colour is a reliable indication of these two types of

oxygenation in the 'A' ring. Colour formation with alcoholic ferric chloride (Briggs and Locker, 1951), colour of the complex formed with aluminium chloride; play of colours, if any in alkaline solutions are some of the other diagnostic colour tests used in the characterisarion of flavonoids. Flavonoids can also be detected by the deep yellow colours formed on treatment with ammonia, a test which can also be used to detect these compounds on paper chromatograms.

CHROMATOGRAPHIC EXAMINATION OF FLAVONOIDS

For a parcticing taxonomist wishing to use flavonoids as chemotoxonomic markers, perhaps, the most convenient and at the same time, quite reliable method is paper chromatography. One advantage of this technique is that it can also be used as a field-test as it does not require any elaborate equipment or sophisticated apparatus. It is possible to rig up a handy and portable chromatographic kit for this purpose. Harborne (1973) and Markham (1982) have suggested useful step-wise procedures for chromatographic examination of crude plant extractives likely to contain flavonoids. Both unidimensional (descending, ascending, and horizontal modes) and two-dimensional paper chromatographic techniques are used; the two dimensional technique is more useful because of its better differentiating power. It is also advisable to run the chromatograms in as many different solvent systems as possible in order to differentiate closely related compounds. The developed chromatogram can be viewed under an ultraviolet lamp, with or without exposure to ammonia. Several spray reagents are also available and these have considerable diagnostic value. For example, 5% ethanolic aluminium chloride gives bright yellow fluorescent spots with 5-hydroxy flavonoids. Other ueful reagents are alcoholic ferric chloride, vanillin-HCl and diazotised sulphanilic acid. Standard Rf value data are available (see, for example, Mabry *et al.*, 1970) but it would be desirable to make direct comparisons with authentic samples. It would be a good idea to keep in the kit-box authentic samples of commonly occurring flavonoids such as kaempferol (11), luteolin (12), quercetin (13), gossypetin (14), quercetagetin (15) and their glysosides. Preparative paper chromatography and thin-layer chromatography can be used for the isolation of small quantities of flavonoids for further characterisation, for example, by spectroscopic methods. HPLC is a very sensitive chromatographic technique which is now being increasingly used in the analysis of flavonoids in crude or partially fractionated extracts. Hostettmann and co-workers (1984) used this technique for the detection of flavonoids and xanthones in crude *Gentiana* extracts. In our laboratories, this technique has been used for a comparative study of the flavonoid glycosides in five species of *Spermacoce* (Krishnaswamy and Nageswara Rao, unpublished observations; Nageswara Rao, 1988).

DISTRIBUTION OF FLAVONOIDS

The occurrence of flavonoids in the plant kingdom is very wide-spread, cutting across a large cross-section ranging from bryophytes and tracheophytes to

angiosperms. Till now, these compounds have not been detected in bacteria and algae. There are distinct and significant differences in the types of flavonoids which occur in primitive forms of plant life as compared to more highly evolved plant species (Markham, 1988). For example, biflavones which are conspicuous in gymnosperms (Niemann, 1988) are comparatively rare in angiosperms (Gianassi. 1988). Flavonoid C-glycosids are also more common in the more primitive forms though they have also been detected in several angiosperms. Thus, while differences do exist, there is no clear cut demarcation line and what is discernible is a gradual transformation of type with evolution. We shall come back to this aspect in a later section.

FLAVONOIDS AS TAXONOMIC MARKERS

Harborne (1975) has summarised the several factors which are responsible for the selection of flavonoids as chemotaxonomic markers. As already mentioned, the wide distribution of these compounds in the plant kingdom, the ease with which they can be detected even in crude extracts, and the availability of simple but reliable methods for their detection, isolation if necessary, and characterisation are the main favourable factors. Several investigators have, therefore, used flavoniods as taxonomic markers and a number of useful results have emerged from their studies. A few of these, which have made significant impact on the progress of plant systamatics are discussed below.

Among lower plants, liverworts and mosses produce flavonoids whereas they are absent in algae and the Anthocerotales. Of the 7000 and odd well-authenticated liverworts, only about 400 species have been examined for their flavonoid content which includes C- and O- glycosides of flavones, flavonols and dihydrochalcones. The distribution of these compounds is uneven; Mues et al. (1986) have pointed out that while more than 70% of the species in the Marchantiales/Sphaerocarpales contain detectable levels of flavonoids, only 38% of the Jungermaniales and 27% of the Metzgeriales seem to elaborate these compounds. Some significant traits have been noticed with each of the different orders. For example, while the distribution of flavonoids in the Jungermaniales is not uniform, the major type, where they occur, is the C-glycoside often having complex structures. In the order Marchantiales, flavone O-glucuronides occur in almost all the families, with just one exception, namely, *Corsinia*. The flavonoid profiles of the Sphaerocarpales very closely resemble those of the Marchantiales with one significant difference, namely, the absence of flavone C-glycosides in the Sphaerocarpales. Markham (1980) showed that on the basis of its flavonoid composition, the minute, desert liverwort, *Carpos* could be conveniently placed in the order Sphaerocarpales; its exact taxonomic position was, earlier, a matter of dispute. In ferns, the flavonoid distribution is very wide-spread and it is not possible to go into a detailed description of it in this brief review. Markham's (1988) review, which includes a 15-page table listing the ferns which have been examined for their flavonoids, is invaluable as a source of information of this subject.

The characteristic flavonoids of the gymnosperms are the biflavones, proanthocyanidins and, to a lesser extent, flavone C-glycosides. Erdtman (1963) made very good use of the flavonoid chemistry for taxonomic classifications of the pines and showed that the subdivision of the genus *Pinus* into Haploxylon and Diploxylon pines, as suggested by morphological studies, was, indeed, supported by the flavonoid profiles of the heartwood of this genus. Members of three families of the order Gnetales do not seem to have the biosynthetic capability for producing biflavones, which are otherwise widely distributed among the Gymnosperms. Niemann (1988) has pointed out that the inability to synthesise biflavones is matched by the capacity to produce, instead, flavone C-glycosides.

Among the several reviews available on the distribution of flavonoids in angiosperms, the most recent is that by Gianassi (1988). The structural variety among angiosperm flavonoids is, indeed, amazing. These include anthocyanins, acylated anthocyanins, flavones, flavonols, their partial methyl ethers, their O- and C-glycosides, proanthocyanidins etc. The overall flavonoid chemistry is in general agreement with the classification scheme of Cronquist (1981). There are, certainly, a few apparently anomalous features which, perhaps, would get clarified when more secondary metabolite characters are taekn into consideration. By and large, however, it is gratifying to note that the correlations between chemical and non-chemical data are of a high order (Gianassi, 1988). Data on flavonoids of the Monocotyledons are not adequate enough to permit firm conclusions to be drawn concerning any possible correlation with flavonoid profile and evolutionary trend (Williams and Harborne, 1988). The situation with regard to the Dicotyledons is much more satisfactory (Gianassi, 1988). The flavonol, myricetin (16), which is usually associated with primitive character, is, surprisingly, absent in the Magnoliidae (Gianassi, 1986). On the other hand, this eompound is quite common in plants of the Hammelidae, Rosidae and Dilleniidae which are considered to be derived from Magnoliidae. The flavonoid profiles in the Hammelidae. Rosidae and Dilleniidae resemble each other and are intermediate between those of the Magnoliidae and the Asteridae. The family Asteraceae shows close relationship in its flavonoid profiles to the Rubiaceae, some Lamiales and Scrophulariales. One classical example where flavonoid chemistry could solve a difficult taxonomic problem is that of the classification of the family Ericaceae. The difficulty here was due to the family's variable vegetative habit. Drue's early and extensive classification of this family was later found to be unsatisfactory when a larger number of morphological characters were taken into consideration (Stevens, 1971). A revised classification based on flavonoid distribution in this family was compatible with the collated, total morphological data (Harborne and Williams, 1973).

FLAVONOID CHEMISTRY AND EVOLUTIONARY TRENDS

It is the considered opinion of taxonomists that herbaceous plants are evolutionarily more advanced than those which are woody in character

(Cronquist, 1968, 1981). Extensive studies have shown that herbaceous plants produce more of flavones than flavonols while the reverse is true of woody plants (Harborne, 1975). Woody plants are also richer in proanthocyanidins which are rare, though not absent, in herbaceous plants. Other structural parameters associated with evolutionary advancement are 6-oxygenation and partial O-methylation. Biflavones and flavone-C-glycosides are, on the other hand, indicators of primitive character. Therefore, flavonoid distribution patterns are useful in diciding whether a particular plant family is more advanced than another and to arrange within the same family the various genera in the order of phylogenetic advancement. An example which brings out the success of this approach is the following. The large family Umbelliferae consists of three sub-families, namely, Hydrocotyloideae, Saniculoideae and Apioideae in the order of evolution. More than 200 plants chosen at random from all the three sub-families were examined for their flavonoid campositions (Harborne, 1971). This included 23 species of the Hydrocotyloideae, none of which was found to contain flavones; they only had flavonols. At the other extreme, of the 243 species of the Apioideae, as many as 79 plants contained both flavones and flavonols. Further, the various tribes of this subfamily could be arranged in the order of increasing evolutionary advancement on the basis of flavone - flavonol ratio. within the Saniculoideae 28 species resembled members of the Hydrocotyloideae in not producing any flavones.

It should, however, be noted that clear-cut inferences regarding evolutionary trends from chemical data which do not contradict indications from non-chemical data are not possible in a few cases. For example, as mentioned earlier, the flavonol, myricetin is absent in the order Magnoliidae but present in Hammelidae though the latter is considered to be derived from the former. However, it should also be noted that in the Magnoliidae, flavonols are more abundant than flavones and this is compatible with the primitive character of the order. Obviously, for reasons not yet clear, aberrations may occur obscuring the value of any one set of characters, in determining evolutionary polarity. A more meaningful correlation would emerge when a larger number of chemical characters are analysed. Ultimately, it should be possible at some future date (hopefully not far away!) to use biochemical characters such as the enzyme and nucleic acid chemistry to sort out difficult taxonomic problems.

CONCLUSIONS

By and large, flavonoid chemistry has proved to be an useful tool to the plant taxonomist. The deficiencies in their use, at present, are due to the largely empirical nature of the application. This, in turn, is a result of lack of knowledge of the precise biochemical functions of these compounds and the rationale for their biosynthesis. Once these aspects are understood, it should be possible to link up structural variations with evolution in a more logical manner. Simultaneously,

information on the enzyme systems involved in the production and metabolism of flavonoids should provide additional valuable input.

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