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COUMESTROL- AND 4',7-DIHYDROXYFLAVONE CONTENT IN FOUR ALFALFA CULTIVARS AFTER OZONE EXPOSURE

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Seven-week old alfalfa plants of four different cultivars were exposed to 773 µg/m^3 (0,40 ppm) ozone for three hours to investigate whether coumestrol is also produced in other legumes after ozone exposure. 48 h after exposure, the most severely injured leaves were harvested for analysis.

Leaves were extracted in methanol and samples were subjected to thin layer chromatography with subsequent fluorometer readings. Coumestrol was never detected in any crude extract of control or experimental samples. However, all crude extracts from plants exposed to ozone contained high amounts of the flavonoid compound, 4',7-dihydroxyflavone. This compound was not detected in any crude extracts from the control plants.

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Abstract

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Introduction

In a study conducted by Hurwitz (22), one alfalfa cultivar, Medicago sativa L. 'Buffalo', was used to investigate the influence of ozone on concentration of the isoflavonoid coumestrol in the foliage. Since alfalfa is a major feed of livestock in the United States and since coumestrol, as a plant estrogen, has been reported to be important for the food quality of alfalfa, the hypothesis that ozone can cause an increase in coumestrol content in alfalfa foliage was tested. No increase in coumestrol content was ever detected. However, another flavonoid compound, 4',7-dihydroxyflavone, was detected after ozonization. As injury severity increased, concentration of 4',7- dihydroxyflavone in the foliage increased as well.

In the present study the ability of four cultivars of alfalfa other than 'Buffalo' to produce coumestrol after ozone exposure was investigated. The degree of 4',7- dihydroxyflavone accumulation in these varieties was also investigated.

The study is part of an extensive research project to correlate effects of air pollutants on plant foliage with qualitative changes in plant organs which constitute the food commodities.

Background

Ozone is regarded to be one of the most serious phytotoxic air pollutants in the United States (24) . Nitrous oxides and hydrocarbons, essential for ozone formation, originate primarily from car exhaust, i.e. incomplete combustion, combustion plants and chemical industries in urban areas. Recently it has been demonstrated that ozone can be transported over long distances (5) and consequently has the ability to accumulate in rural agronomic and forested areas. In field investigations and in the laboratory ozone effects on foliage (necrosis), effects on yield and on different food and feed quality parameters have been demonstrated. Because of ozone's ability to occur in phytotoxic concentrations even in rural areas and because of the injury to plants which manifests itself in economic losses it is necessary to fully understand the total impact of ozone on vegetation.

Alfalfa, Medicago sativa L., is a major feed of live-stock in the United States. It is sensitive to ozone as manifested by macroscopic foliar injury (7, 18). Another result of ozone exposure is yield reduction (17, 33). However, changes in quality of alfalfa foliage due to ozone exposure have not yet been examined as thoroughly. Since other stresses including climate, nutrition and diseases can affect alfalfa quality (14) it seemed appropriate to also consider the impact of ozone on some qualitative parameters.

Flavonoids are compounds which, in too high concentrations, can alter the quality of alfalfa. Flavonoids are phenolic compounds which represent a widespread group of water-soluble compounds, mainly colored red, purple or yellow. Many types of flavonoids are found in one plant and these are natural constituents in all vascular plants (Table 1). Flavonoid compounds normally exist in plants as phenolic glycosides. The sugar free phenolic moieties are referred to as aglycones. In healthy plants, these are present only as traces.

The most common basic flavonoid skeleton, shown in Figure 1, is usually modified in such a way that more double bonds are present, causing the compounds to absorb visible light and thus giving them colour. The two carbon rings at the left and right ends of the molecule are designated the A and B rings, respectively. Hydroxyl groups are usually present in the flavonoids, often making them sufficiently water-soluble to accumulate in the cell vacuoles. All flavonoids are soluble in methanol or ethanol. Flavonoids are partially products of shikimic acid pathway. Ring B (Figure 1) and the carbon atoms of the central ring are formed from shikimic acid and thus from phenylalanine or tyrosine. Ring A is probably produced from head-to-tail condensations of acetate units. It has been suggested that acetyl Co A is converted to malonyl Co A, and that three of these malonyl Co A molecules condense with each other and with a derivative of a phenolic acid, such as p-coumaric, forming the basic flavonoid structure. Flavonoid content in plants varies with light, temperature, water conditions, availability of minerals, stage of growth and genetic differences (15). As a rule, the flavonoid contents increase when plants are stressed by biotic diseases (26, 32, 3, 40, 35) or by abiotic influences including chemicals, physical wounding (35), adverse temperatures, high soil moisture and low nutrient levels (13, 38). If the flavonoids which become elevated as a result of these stresses have deleterious properties, coumestrol is estrogenic; for example, any induction of such a compound would reduce the quality of the crop.

The two flavonoids dealt with in this study were 4',7-dihydroxyflavone (4',7-DHF), a flavone, and coumestrol, an isoflavonoid (Figure 2).

4',7-DHF was first isolated and identified from healthy alfalfa plants in 1965 by Bickoff et al. (1) (Figure 1 and 2). Little is known about the function of 4',7-DHF in plants. However, 4',7-DHF has been shown to accumulate as an aglycone, in response to pathogenic fungal infection in alfalfa (32, 3).

4',7-DHF production is also induced in detached alfalfa shoots treated with abiotic stresses such as copper chloride or cadmium chloride (35).

Coumestrol belongs to the isoflavonoid group (Figure 1 and 2). Bickoff et al. (2) isolated and identified coumestrol in alfalfa in 1957. Coumestrol can exist as an aglycone in trace amounts in healthy tissue of alfalfa (3). However, if alfalfa becomes infected with pathogenic fungi, coumestrol production is induced (35).
In contrast to other flavonoids, which as a whole are harmless substances, isoflavonoids have estrogenic, insecticidal and fungicidal properties. Isoflavonoids also have a very limited taxonomic distribution. Most are found in the sub-family Lotoideae of the Leguminosae and occasionally in the sub-family Caesalpinioideae and in a few other families, e.g. Rosaceae, Moraceae, Amaranthaceae, Iridaceae and Podocarpaceae. Isoflavonoids are weak estrogens and their presence in forage legumes, such as alfalfa (Medicago sativa L.), subterranean clover (Trifolium subterraneum L.) and red clover (Trifolium pratense L.) has shown to be both beneficial and detrimental. Elevated levels of isoflavonoids have been associated with increased milk flow and increased growth of cattle (36). However, these compounds have also been associated with fertility dysfunctions in sheep (6) and dystocia and vaginal prolapse in ewes (4).

The possibility that ozone might induce flavonoid synthesis has been studied in a few plant species. Keen and Taylor (25) found that coumestrol, daidzein and sojagol accumulated in soybean foliage in response to ozone-induced injury. An accumulation of anthocyanins has been reported in morning glory (Ipomoea sp.), curly dock (Rumex crispus L.) and poinsettia (Euphorbia pulcherrina Wild.) after ozone exposure (31, 27, 9). Some studies have also been carried out as regards the influence of air pollution on accumulation of other phenolic compounds in plants (19, 21). Howell (20) has reported an increased production of caffeic acid in beans. Menser and Chaplin studied

the effects on phenol and alkaloid content in tobacco leaves (Nicotiana tabaccum L.) after ozone exposure (30). Higher levels of polyphenols were measured in ozone-injured leaves than in healthy tobacco leaves.

Materials and methods

Cultural conditions:
Four different cultivars of alfalfa were studied in the experiment:
Medicago sativa L. 'Moapa', 'Sonora', 'Ladak' and Medicago sativa L. x Medicago falcata L. 'Vernal'.
Seed was sterilized before planting as follows: soaked in 95% ethanol for 10 minutes, soaked in 0,1% mercuric chloride ($HgCl_2$) in 70% ethanol for 30 minutes. Then it was washed in distilled water before planting. "Jiffy Mix", a peat-vermiculite mixture, was used as growth medium. Soluble salts were leached with tap water from the "Jiffy Mix" and approximately 400 seeds were planted per flat (45 x 30 cm), 100 of each cultivar. On each flat two rows of each cultivar were grown, always starting with 'Ladak' on the edge of the flat and then 'Vernal, 'Sonora', 'Moapa', 'Ladak', etc. After germination, seedlings were thinned to 96 per flat, 24 per cultivar. Plants were grown in a greenhouse with supplemental incandescent and fluorescent lighting of 4000 Lux and 15-h photoperiod.
Plants were watered to saturation and fertilized with 3/4 of a teaspoon of "Peters Peat- Lite Special" per 2 liters of water two weeks after seeding and subsequently once a week until ozone exposure. Insects were controlled with a weekly spray of "Resmethrine".

Ozone exposure:
Plants were watered to soil saturation and placed in the exposure chamber 15 hours before treatment and returned to the greenhouse 3-4 hours after ozone exposure. Plants were exposed to ozone in a modified Environmental Growth Chamber, previously described (41). The chamber was maintained at 21^{o}C, 70% relative humidity and a light intensity of 25000 Lux, with a daily 18-h photoperiod, beginning at 06.00 hours. Ozone was produced with an Orec Model 03V1 generator. Ozone concentration was monitored by the chemiluminescent method utilizing a REM Incorporated Ozone Monitor, model 612-B, which was calibrated with 1% potassium iodide solution (23).

Experimental design:
The experiment was repeated three times, designated as trial I, II and III.
Seven week-old plants were exposed in the three trials, to 773 $\mu g/m^3$ (0,40 ppm) ozone for 3 hours. In each trial 170 plants of each cultivar were exposed to ozone and 75 plants of each cultivar were used as control plants. 48 hours after exposure, when injury had developed, the injured foliage was harvested. All leaves with severe and similar injury rating were pooled together for tissue analysis. The leaves that were harvested belonged to injury categories 3 and 4 (3= chlorosis and/or necrosis and 4= extensive necrosis) were harvested according to Hurwitz (22). Data were analyzed by analysis of variance.

Tissue extraction:
Leaves were mixed to ensure random distribution. One gram of foliar samples were taken from the pooled leaves. Control plants were harvested and prepared exactly the same way as the fumigated plants. For each variety of ozonized and control plants ten one-gram samples were taken for dry weight determination. The one gram foliar samples were cut into small pieces and extracted for three days or more in 15 ml of 80% methanol in screw-cap glass vials. Samples were vacuum-filtered through two layers of Whatman No. 1 filter paper. Extracted leaves were washed with two 15 ml portions of 80% methanol and the combined filtrate was flash-evaporated to near dryness, under reduced pressure at $40^{o}C$. The residue was brought to a volume of 2 ml in 80% methanol and these samples are in the following referred to as crude extract.

Analysis of coumestrol and 4',7-dihydroxyflavone

Crude extract samples were subjected to thin layer chromatography (TLC) to determine if coumestrol and 4',7-DHF were present and to quantify the amounts.
From each experiment four replications (crude extract samples) of each variety were analyzed.
Plates were prepared with Silica Gel G (0,25 mm thick). The plates were air-dried and activated in a microwave oven immediately prior to use. Ten samples were spotted on each plate. Authentic coumestrol and 4',7-DHF were used as standards, (0,04 and 0,05 µg/µl methanol respectively, were spotted at volume 10 µl. Ten µl of the crude extract samples were spotted at the nine remaining positions). Plates were developed in two solvent system; hexanes - ethyl acetate - methanol (60: 40:1 by volume) and toluene - ethyl formate - formic acid (5: 4:1 by volume) for approximately one hour and allowed to dry for a minimum of three hours before observation with a fluorometer (42), which was directly connected to a galvanometer.
A background fluorescence reading was obtained from each plate and subtracted from the values to obtain a true quantitative measurement of fluorescense.

Quantification of 4',7-dihydroxyflavone (4',7-DHF) in crude extracts:

Authentic 4',7-DHF was obtained and standard solution was prepared at 0,005 µg/µl methanol and 0,05 µg/µl methanol.
Two, 3, 5, 7, 9, 12, 14, 16 and 18 µl of the 0,005 µg/µl methanol and 1, 2, 3, 4 and 5 µl of the 0,05 µg/µl methanol were spotted on silica gel plates, which were developed in a solvent of toluene - ethyl formate - formic acid (5:4:1 by volume).
Fluorometric readings were then taken from the standard spots. Two runs, consisting of between four and ten spots of each of the fourteen volumes of standard solution were completed.
A fluorometer was utilized to quantify the 4',7-DHF as described above.

A standard curve for 4',7-DHF was prepared from the mean deflection readings of the 14 authentic flavone standard spots. Fluorometer readings of crude extracts were compared with the fluorometer readings of the authentic 4',7-DHF in the standard curve and then converted to parts per million dry weight (ppm). All values were quantified on the basis of this curve.

Injury severity test:

The four cultivars were exposed to 0,40 ppm ozone for three hours as described earlier. Two different experiments were conducted. In the first experiment seven week old plants were exposed to ozone. 48 hours after exposure, when symptoms had developed, the foliage was rated on 180 plants of each cultivar and grouped as follows: 1= no visible injury, 2= slight stipple on abaxial leaf surface, 3= chlorosis and/or necrosis and 4= extensive necrosis. Each plant was grouped according to the most severe injury seen on the foliage and a mean injury severity was received for each cultivar.
In the second experiment ten week old plants were exposed to ozone for three hours. 48 hours after ozone exposure, when symptoms had developed, between 27 and 37 plants of each cultivar were rated. Each leaf of each plant was rated individually and grouped as described above. A mean injury severity was received for each plant and then for each cultivar.

Results

Visible injury was not apparent on all plants following ozone exposure. Dark water-soaked spots were seen on foliage on which injury developed later. 24 hours after exposure, injured leaves exhibited chlorosis or slight necrosis, which later developed into a more extended necrosis. The pattern of injury was variable, sometimes occurring on the leaf margins and interveinal areas in small isolated patches and other times only injuring foliar tissue directly adjacent to the veinal area. Slight injury was characterized as a faint, chlorotic stipple on the abaxial leaf surface.

Coumesterol was never detected in any crude extract of either control or experimental samples from either trial. The lower limit of detection was 5 ppm on a wet weight basis.

4',7-DHF was detected in all crude extracts from ozone-injured alfalfa foliage in all four varieties. Results from the three different trials are represented in Table 2.

There was no significant difference in concentrations of 4',7-DHF between the four varieties. The highest level of 4',7-DHF in an individual sample was 91 ppm (dry weight) in the alfalfa cultivar 'Ladak'.

Control plants contained no detectable 4',7-DHF. The lower limit of detection was 0,4 ppm on a wet weight basis.

The R_f value for 4',7-DHF was 0,45. Several additional, unidentified fluorescent compounds occurred in the area of chromatograms where flavonoids would be expected. In some of the crude extract samples one specifically bright blue fluorescent spot was seen at an R_f of 0,55.

The results of the injury severity test to study differences in susceptibility to ozone between the four varieties are presented in Table 3. 'Sonora' was most susceptible, followed by 'Moapa' and 'Vernal'. 'Ladak' seemed to be the least susceptible. Based on this method to rate injury, no statistically significant differences between cultivars were detected.

Discussion

According to Hurwitz' results (22), plants grown during winter months are much more sensitive to ozone than plants grown during the Summer. Both temperature and light intensity affect plant sensitivity and may be responsible for the increased sensitivity of alfalfa during winter months (12, 16).
The alfalfa plants in this study were grown from October through January. Plants in all three trials exhibited similar severe levels of injury, even if injuries on individual plants varied.

Some differences in ozone sensitivity between the four cultivars were also seen, but they were not statistically significant. As a consequence of the rather high ozone concentration used, we might have overcome possible differential effects. Our results from the test comparing the sensitivity of the cultivars do not agree with the results reported by Howell (20), who found that 'Vernal' is more sensitive than 'Moapa'.

Alfalfa leaves infected by pathogenic fungi accumulate large quantities of coumestrol and related flavonoid compounds (28,29). In a study done by Sheerwood et al. (35) it was shown that coumestrol accumulation was also induced in alfalfa leaves by inoculation with pathogenic bacteria and nonpathogenic fungi. In the same study detached roots and shoots were treated with a copper chloride solution which resulted in a coumestrol accumulation only in the roots. In the same study, alfala leaves were exposed to formaldehyde gas, an atmospheric oxidant, to study effects of another abiotic factor. No coumestrol could be detected in these leaves. Alfalfa leaves of the 'Buffalo' cultivar that were exposed to ozone did not accumulate detectable amounts of coumestrol (22).
In this study we have similarly shown that coumestrol does not accumulate in leaves of the four additional alfalfa cultivars,

even if this has been reported to be the case for soybean, another legume (25). Whether this discrepancy is due to a genetically based difference in metabolism, e.g. differences in the way the plant responds to different biotic and abiotic stresses, is not known today.
However, the fact that coumestrol has been found in trace amounts in apparently healthy alfalfa leaves is interesting (13, 36, 37).

The general estrogenic activity in alfalfa leaves in different development stages of alfalfa has been studied. Keen and Taylor (25), Pieters and Andrews (34) and Bickoff et al. (1) reported a considerable fluctuation in activity at different stages of maturity. An increase in estrogen content in the early budding and flowering stages was followed by a decrease until the fourth bloom, pointing to a variation in plant metabolism with plant age. The alfalfa plants in our experiment were seven weeks old and not yet mature. Therefore we might have detected coumestrol after ozone exposure in fully mature (12-14 weeks old) alfalfa leaves.

In this study we did not find detectable amounts of coumestrol in any tissue. To be sure that the technique used for coumestrol extraction was valid, some samples of fungally infected alfalfa leaves were analyzed. High amounts of coumestrol was found in those leaves. Furthermore, our fluorometer could detect levels of coumestrol concentrations similar to those found in the soybean foliage (25).

The mechanisms behind the induction of flavonoid compounds, as a response to different environmental stresses, has been discussed but is not yet clearly understood. Ohla et al. (32) have suggested that fungal induced accumulation of flavonoid aglycones may involve conversion of pre-existing flavonoid glycosides to aglycones. Possibly glycosidases, necessary for the conversion, are produced by the host in response to the

pathogen, or are actually provided by the plant pathogen. The host may also synthesize aglycones immediately. The final step, the glycosylation, might be impaired by environmental stresses such as ozone. In parallel with this, Keen and Taylor (25) suggested that ozone-induced foliar injury in soybeans operates through invocation of the hypersensitive disease-resistance response. This means that the toxicity associated with stippling and necrosis in ozone-damaged plants may be due to the post-treatment production of flavonoid and other phenolic compounds by the plant. Others have reported that hormones like ethylene and cytokinins control the synthesis of flavonoids (11, 8). Craker (10) and Tingey (39) reported an increased ethylene production from ozone-injured plants. One hypothetical explanation to the mechanisms of induction of flavonoid synthesis in ozone-exposed plants might therefore be an increased ethylene production.

It has been demonstrated that 4',7-DHF, unlike coumestrol, can be induced in intact alfalfa leaves in response to both biotic and abioitic stresses such as copper chloride and cadmium chloride (35). Hurwitz (22) found high amounts of 4',7-DHF in the 'Buffalo' cultivar in response to ozone exposure. The ozone induced injury of the alfalfa leaves correlated well with the 4',7-DHF-concentrations of the same leaves. Our results agree with Hurwitz' as far as the 4',7-DHF induction in ozonized alfalfa leaves is concerned. However, we never detected as high concentrations in the alfalfa leaves. Furthermore, visible injury was necessary for induction of the compound.

In this study no 4',7-DHF was found in the leaves of the control plants. In contrast to this, some researchers have found 4',7-DHF in healthy or apparently unstressed alfalfa leaves. One possible explanation to these different results might be that the alfalfa plants in our study were cultivated differently from the study done by Hurwitz (22), where the plants were grown individually in small pots. This small pot could cause a certain 4',7-DHF-inducing stress to the plant, compared to the plants growing

in a big flat with more soil as in our case. Even if our types of injury on the leaves agreed, we did not find the same amounts of 4',7-DHF. A genetical difference between 'Buffalo' and the other four cultivars seems to be a less probable reason.

The biochemical and physiological events responsible for 4',7-DHF accumulation during ozone exposure are not yet well defined. One speculative suggestion is that plant glycosides are ruptured by an increased amount of glycosidases enzymes, leading to increased aglycone concentration; another is that ozone impairs glycosylation at the final steps of flavonoid biosynthesis. The third suggestion is that an aglycone synthesis is started as a response to different stresses as a general resistance reaction. Not much is known about the function of 4',7-DHF or its possible modification of the quality of alfalfa. It is, however, remarkable that much greater concentrations of the flavonoid 4',7-DHF occur both in photochemically injured foliage of alfalfa, due to an abiotic stress, and in foliage infected by plant pathogens, due to biotic stresses.

Table 1

Flavonoids
Anthocyanins
Flavones (4',7-dihydroxyflavone)
Flavonols
Flavone and flavonol glycosides
Chalcones, aurones and dihydrochalcones
Natural proanthocyanidins
Flavanones and dihydroflavonols
C-Glycosylflavonoids
Biflavonoids
Isoflavonoids(Coumestrol)
Neoflavonoids

Table 2

Concentration of 4',7- dihydroxyflavone (ppm dry weight) in alfalfa foliage from four cultivars, 48 h after exposure to 773 µg/m^3 (0,40 ppm) ozone.

	Trial		
	I	II	III
Control (L, S, V, M)	N.D.	N.D.	N.D.
	sd	sd	sd
Ladak	60 (±16)	50 (±19)	42 (±17)
Sonora	41 (±16)	64 (±28)	35 (±11)
Vernal	43 (±18)	47 (± 3)	46 (±19)
Moapa	44 (±20)	53 (±15)	47 (±20)

Table 3

Injury severity test of four alfalfa cultivars: 'Moapa', 'Sonora', 'Vernal' and 'Ladak'.

1= no visible injury 2= slight stipple on abaxial leaf surface

3= chlorosis and/or necrosis 4= extensive necrosis

Two experiments, A and B, were conducted.

The ozone doze was 0,40 ppm during three hours.

Variety	Experiment	
	A [a]	B
Ladak	2,53 ± 0,40	2,20 (28)[b] ± 0,58
Vernal	2,60 ± 0,38	2,44 (35) ± 0,50
Moapa	2,99 ± 0,33	3,13 (27) ± 0,56
Sonora	3,10 ± 0,32	3,23 (37) ± 0,57

a) injury severity average of 180 plants of each variety

b) number of plants

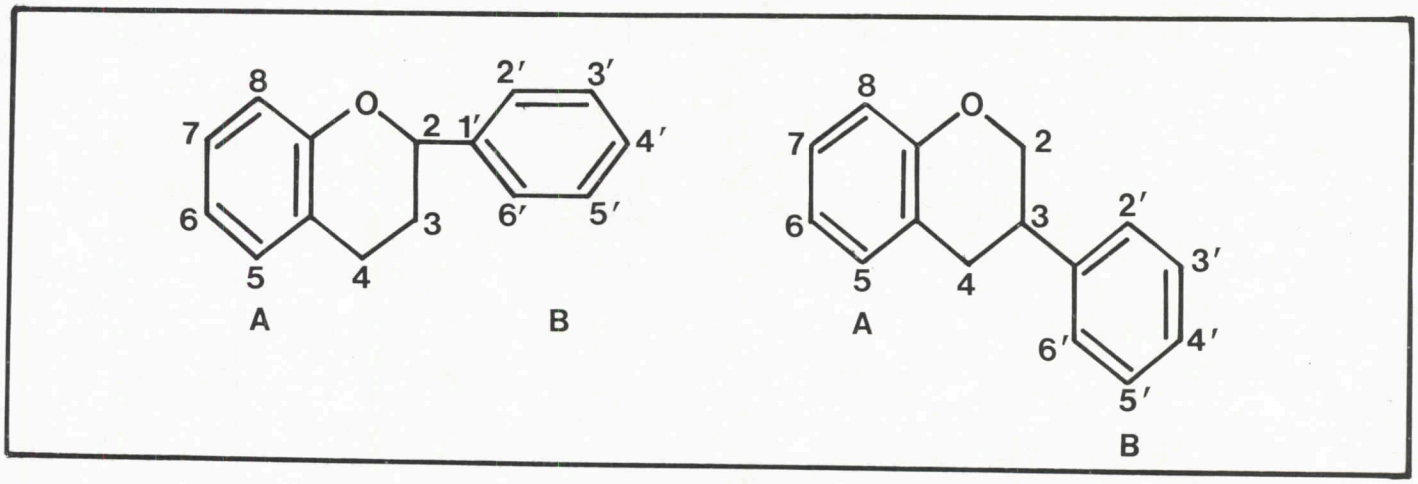

Figure 1

Flavonoid and isoflavonoid skeleton

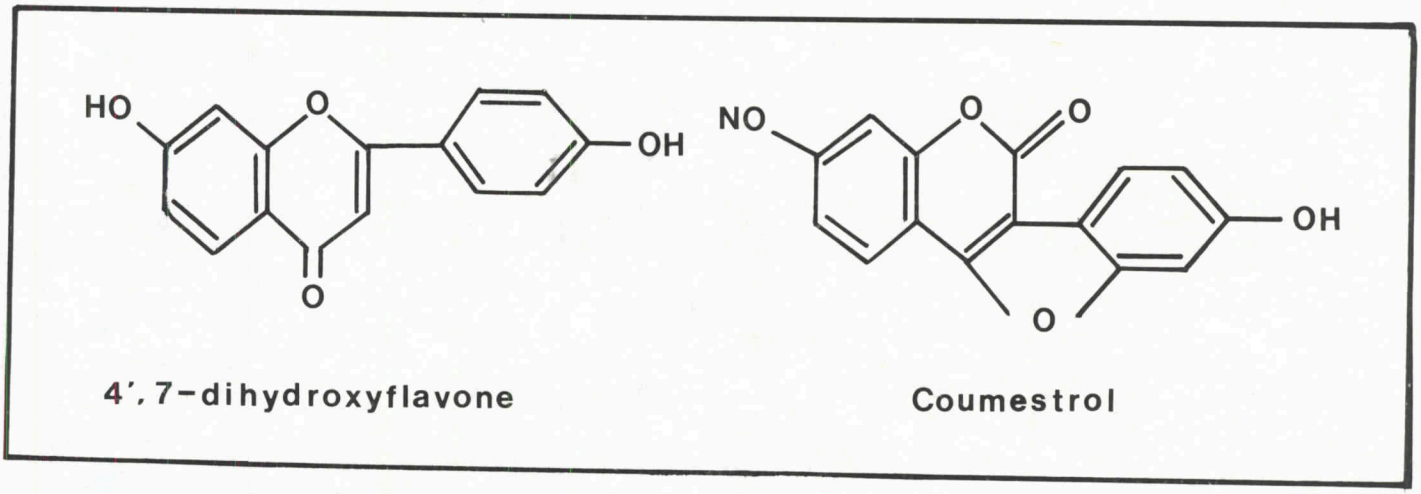

Figure 2

Chemical structure of 4',7-dihydroxyflavone
and coumestrol

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