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Review Article

"IMMUNOMODULATORY AND ANTIOXIDANT ACTIONS OF DIETARY FLAVONOIDS"

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ABSTRACT

Flavonoids are natural substances that are present in vegetables and fruits. Nearly 4000 different flavonoids have been identified till date and they constitute a major part of our daily diet. Flavonoids are classified into different categories based on their chemical structure as flavanones, flavones, flavonals, catechin, isoflavone, chalcones and anthocyanin. It has been discovered that they have many beneficial actions on body cells by enhancing the activity of many enzyme systems. The aim of this present review is to summarize the significant and recent advancements in the field of immuno-modulation and anti-oxidant actions of dietary flavonoids.

Keywords: Flavonoids, Immunomodulatory, Quercetin, Inflammation, Anti-oxidants.

INTRODUCTION

Various fruits and vegetables contain substances known as "phytochemicals" that are beneficial for the human body to prevent acute and chronic diseases [1]. Phenolics are important group of phytochemicals belonging to the class polyphenols which are very important secondary metabolites in plants [2]. Based on the carbon skeleton, the classification of the polyphenols is as: phenolic acids and flavonoids.

Bioavailability of Flavonoids

Flavonoids are bioactive polyphenols of low molecular weight [3,4,5], that play an important role in photosynthetic plant cells [6]. Flavonoids are identified by their flavan nucleus [7] and carbon skeleton [8,9]. Until nearly 50 years ago, the mechanism of flavonoid action was not clear. The basis of research in flavonoids began in early 1930s; Albert Szent- Gyorgi a Hungarian scientist, discovered a new substance from oranges. It was first termed as "Vitamin P". Later, it was clarified that this substance was " Rutin" (a Flavonoid), Hence large number of research on flavonoids started.

Structure of flavonoids

The flavonoids (Fig.1) contain two benzene rings linked by a pyran heterocyclic ring [6] [2 - phenyl benzo gamma pyrane nucleus].The arrangement of flavonoids in their methoxy, hydroxyl, glycosidic groups and in the conjugation between A ring and B rings vary [4]. Many subclasses of flavonoids are found with the difference in the C ring (Tabe 1 and Fig.2).

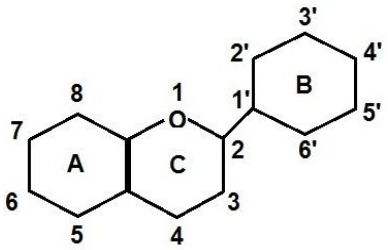

Fig. 1: General Structure of Flavonoids [6]

Table 1: Different subclasses of flavonoids and their sources [5, 10]

S. No.	Sub-classes of flavonoids	Important flavonoids present	Source
1.	Flavonones	Naringen, Herpesitin	Citrus peels
2.	Flavonol	Quercetin, Rutin	Tea, Onion
3.	Catechins	Catechins, Epicatechins	Red wine, Tea, Fruits
4.	Isoflavones	Daidzen, Glycindin	Beans
5.	Flavones	Apigenin,Luteolin, Quercetin	Citrus fruit, Parsley
6.	Anthocyanidins	Cyanidin,Delphinidin, Malvidin	Grapes, apple skin, celery, Berries, Olives, grapes, Tea.

Flavonoids are usually present in plants as aglycones (without attached sugars), glycosides (O- Glycosides or C-Glycosides) and methylated derivatives. O-Glycosides contain sugar attached to the hydroxyl (–OH) group of aglycone, whereas C-glycosides contain sugar moiety attached to the Carbon (C) of aglycone.

Flavonoids- distribution, conjugation, absorption and toxicity

Distribution of Flavonoids

Flavonoids are found widely in plants. They are an important part of the daily diet [11, 12,13]. Studies showed that the dietary intake of flavonoids on an average is 1-2g/day [3]. The average intake of quercetin was found to be 16mg/day and that of flavonols and flavones 23mg/day [14]. The distribution of various flavonoids in different medicinal plants in India is shown in Table 2.

Conjugation of Flavonoids

Flavonoids primarily conjugate with the glucoronide moiety of the intestinal cells, following this it binds with albumin for its transporatation to the liver [15, 16]. Further, conjugation is extended by the liver by adding a methyl group, sulphate group or both. These mechanisms decrease toxicity and increase the circulatory time of the flavonoids. There are many potential sites for the conjugation.

The different types of conjugations on the flavonoid skeleton influence the enzyme inhibiting potential and the anti-oxidant property of the flavonoid. Evidences showed that occasional intake of individual flavonoids and their conjugates may not increase the concentration in blood. However studies also suggested that the conjugated flavonoids have longer half lives (23-28h) and hence accumulation can occur at regular intakes which in turn may result in active flavonoid concentrations.

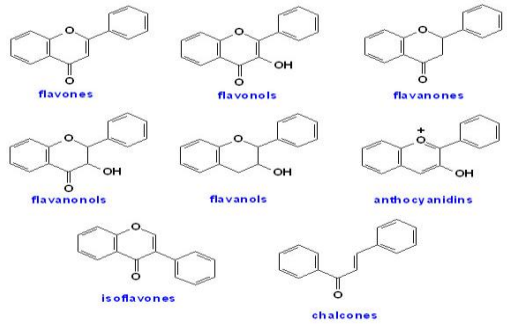

Fig. 2: Molecular structure of different types of flavonoids [5]

Absorption of Flavonoids

Available literature and data on the adsorption, conjugation, metabolism and toxity of flavonoid in humans is scarce [17,18]. Few studies showed that flavonoid quercetin is absorbed by the human body in sufficient amounts [17, 19]. Most of the natural flavones exist in the glycosylated form than the aglycone form. The "form" that the flavonoid exists in was found to influence the rate of adsorption. Studies by Hollman and Katan [20] suggested that quercetin in glycosylated state was quickly absorbed than the a-glycone state [18]. However for catechin vice-versa was seen [21].

Toxicity of Flavonoids

Evidences suggest that flavonoids are non-toxic to normal cells and are toxic to immortalized or cancer cells. Studies by Dunnick *et al*.,reported that increased doses of quercetin over a long period results in tumour formation in mice [22]. However in other studies involving several years no cancer was evident [23].

Many recent literatures suggest many flavonoids like quercetin seem to act as anti-cancer agents *invivo* [24, 25,26]. Studies by Knekt *et al*., involving 9959 women and men for 24 years showed the relationship between consumption of flavonoids and cancer [27].

Table 2: Distribution of flavonoids in few Indian plants

S. No.	Plant	Flavonoid present	Reference
1.	*Acalypha indica*	Clitorin,Biorobin, Nicrotiflorin and Kaempferol	[28]
2.	*Aloe vera*	Luteolin	[29]
3.	*Andragraphis paniculata*	Methoxy and tetramethoxy flavones	[30,31]
4.	*Azadirachta indica*	Kaempferol, myricetin and quercetin	[32,33]
5.	*Bacopa moneirra*	Luteolin	[28]
6.	*Bauhinia monandra*	Quercetin	[34]
7.	*Betula pendula*	Quercetin,Kaempferol, Myricetin	[35]
8.	*Brysonima crassa*	Amentoflavone, Quercetin–3-O-d-galactopyranoside	[36]
9.	*Butea monospermea*	Genistein, Prunetine	[37]
10.	*Calendula officinalis*	Quercetin	(35)
11.	*Citrus medica*	Hesperidin,Naringin, Eriocitrin	(38)
12.	*Glyccheriza glabra*	Liquiritin, Isoliquiritin	(35]
13.	*Limnophila indica*	Skullcapflavone	[36]
14.	*Mentha cogifolia*	Luteolin, kaempferol	[37,38]
15.	*Mimosa pudica*	Isoquercetin, Apigenin	[36]
16.	*Tephorsia purpurea*	Purpurin,Pongamol, Karanjin, Lanceolatin-B	[36]
17.	*Pongamia pinnata*	Pongaflavonol,Pongachin, Luteolin	[37, 38,39]

Anti-oxidant activity of flavonoids

Reactive Oxygen Species(ROS) and free radicals are continuously produced in the body (Table 3) during the normal metabolism of oxygen or induced by any toxin exogenously [40,41].Studies revealed that there are many mechanisms by which free radicals interfere with normal cellular function. One of the most important mechanisms exerted by these free radicals upon cells is "lipid peroxidation", which leads to cellular damage of the cell membrane, followed by a change in osmotic pressure that results in cell swelling and cell death. The most remarkable property of flavonoids is their anti-oxidant activity. Previous studies have revealed that the catechins and flavones were found to be the most potential flavonoids for defending the body against free radicals and ROS. Living organisms have several remarkable mechanisms for protecting themselves from free radical or ROS damage [42]. These include the antioxidant cascade of enzymes such as catalase, glutathione peroxidase, superoxide dismutase, non-enzymes such as ascorbic acid, alpha-tocopherol and glutathione (Endogenous antioxidants). An increased formation of ROS / free radicals leads to depletion of endogenous antioxidants. Flavonoids effectively act as anti-oxidants by two methods. First, they can increase the ability of endogenous anti-oxidants. Secondly, they can interfere with three different ROS/Free radical producing systems in the human body.

(i)Direct Scavenging of free radicals

Flavonoids directly act upon free radicals to stabilize the free radicals by acting with the most reactive compound of the free radical. The radicals are made inactive by the high reactivity of hydroxyl group present in the flavonoids.

R· + Flavonoid (OH) = RH + Flavonoid (O·)

Where, O· is the oxygen free radical and R· is the free radical. Example, flavonoids such as rutin and epicatechin are potential radical scavengers (43).Few specific flavonoids can directly act on superoxides and scavenge them, whereas others can scavenge the ROS derived radical termed as "peroxynitrite".

(ii) Nitric oxide (NO) radical scavenging

Cells such as macrophages and endothelial cells produce NO at high concentrations when under stress hence resulting in oxidative damage. Under such circumstances, macrophages also increase the production of superoxide anion along with NO. The produced NO and free radicals interfere with each other inside the cells and lead to the formation of highly toxic "peroxynitrite", which in turn damages the lipid cell membrane irreversibly.

In such cases, the presence of flavonoids scavenges the produced free radicals [44] resulting in less cellular damage and also it was reported that flavonoids can also directly act upon NO radical itself [45]. Studies by Dehmlow *et al*., showed that silibinin, a flavonoid inhibited NO activity in a dose dependent manner [46].

(iii) Xanthine oxidase

The metabolism of xanthine takes place as follows,

Xanthine Uric acid ⟶ Xanthine

This pathway was found to be an important route in the oxidative damage to tissues, especially after ischemic-reperfusion [47].During reperfusion, xanthine oxidase acts with molecular oxygen hence releasing free radicals.

Studies revealed that flavonoids such as silibin and quercetin inhibit the activity of xanthine oxidase, thereby preventing oxidative damage [48, 49, 50].

(iv) Lipid peroxidation by free radicals

Iron in the presence of free radicals leads to lipid peroxidation [51]. Studies revealed that specific flavonoids such as quercetin were found to chelate iron and hence prevent lipid peroxidation [52, 53].

(v) Leucocyte Immobilalization by free radicals

Under general environmental conditions leukocytes move around freely near the endothelial wall. In contrast to this during inflammation, the complements and other mediators of inflammation lead to the immobilization of leukocytes to the endothelial walls, hence leading to the formation of free radicals. This results in tissue damage and injury.

Studies by Friesenecker *et al* showed that administration of oral dose of pure micronized fraction of flavonoids was found to reduce the leukocyte immobilization during reperfusion [54].

This may be due to the action of flavonoids on the total serum complements and hence act as a protective mechanism against inflammation [55].The remarkable antioxidant capacity of flavonoids were reported by various group of scientists [56, 57, 58, 59, 60, 61]. The summary of the action of flavonoids on different diseases in shown in Fig.3.

Table 3: Different mechanisms by which ROS are produced inside the body [15]

S. No.	Reactive species	Mechanism
1.	Superoxide anion (O_2).	Reduction product of O_2 (One electron reduction), generated by heme proteins.
2.	Hydrogen peroxide (H_2O_2).	Reduction product of O_2 (Two electron reduction)
3.	HO_2	Formed by the addition of proton to O_2
4.	OH (Hydroxy radical)	Generated by "Fentons reaction". It is formed by the reduction of O_2 (3 electron reduction of O_2).
5.	$^1O_2$	Singlet oxygen production
6.	ROO (Peroxy/ Lipid Peroxy radical)	Produced by proton hydrogen abstraction
7.	RO (Alkoxy radical)	Produced by organic hydroperoxide

Flavonoids as immunomodulators and anti- inflammatory mediators

"Inflammation" is defined as the combined response of many protective systems of the body against the action of a "Foreign substance". "Inflammation" involves various mediators and processes such as tissue hormones, cytokines, complements in serum, blood coagulation, cellular and humoral immunity, repair processes and angiogenesis. It is a free radical generating process. The action of flavonoids as immunomodulators is tabulated in table 4.

(i) Inibition of Cyclooxygenase/Lipoxygenase Pathway by Flavonoids

The production and release of the compound Arachidonic Acid (AA) is involved in the initial phase of inflammation. Lipoxygenase (LOX) and cyclooxygenase (COX) play a significant role in the inflammatory process. They are important for the release of "arachidonic acid". Studies revealed that chemotactic substances are formed from arachidonic acid by the lipoxygenase containing neutrophils. This cascade of events also leads to the release of cytokines (Fig 4) such as interleukins [62].

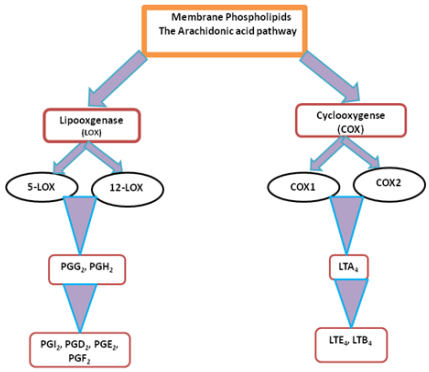

Fig. 3: The Arachidonic acid (AA) pathway [65]

Studies showed that selected phenols were found to inhibit the action of cyclooxygenase and lipooxygense pathyways [62, 63, 64] and modulate their actions. The phenols act by inhibiting the release of arachidonic acid [65]. Studies involving quercetin showed that it inhibited both the lipooxygenase and cyclooxygense pathways, thus inhibiting the formation of inflammatory mediators [66].

(ii)Inhibition of Eicosanoid Pathway

Prostaglandins (PGs) are eicosanoids that are mediators involved in inflammation [67]. It was investigated and found that flavonoids inhibited the eicosanoid biosynthesis pathway [68]

(iii) Immuno-modulation of cell functions related to inflammation

Various flavonoids show a significant pharmacological and biochemical activity that effect the normal functions of immune cells such as B- cells, T- cells, macrophages, neutrophils, basophils and mast cells [69].

Enzymes such as tyrosine and serine protein kinases are mainly involved in inflammatory processes susch as T cell proliferation and cell activation [70, 71] or cytokine production [72]. Flavonoids such as genistein were found to modulate such enzymes. Genistein also prevented the enzyme activity of T-cell specific protein kinases, p561ck and hence decreased IL -2 and IL-2B production. Quercetin was found to prevent enzyme activity of elastase [73] and hence prevent neutrophil degranulation. Other flavonoids such as apigenin, Kaempferol, luteolin and quercetin have shown to inhibit the enzyme beta-glucoronidase and hence lysozyme release from the neutrophils. These flavonoids also prevented arachidonic acid release and hence inhibited degranulation [74].

(iv) Immunomodulation of pro-inflammatory enzyme activities

Various flavonoids were found to modulate the activity of metabolic enzymes involved in AA pathways. These enzymes include COX, LOX, Phospholipase A_2 (PLA_2) [75, 76], Nitric oxide (NO) and NO synthase (NOS). The prevention of these enzymes decreases the formation of AA, Leucotrienes, NO and prostaglandins that are all significant mediators of inflammation.

Thus the inhibitions of such enzymes are important contributors for the "anti-inflammatory" activity of flavonoids. The various immuno-modulatory studies of flavonoids are tabulated in table 5.

Table 4: Summary of immuno-modulatory mechanisms of flavonoids

S. No.	Action of flavonoids	Mechanism of action	Effect of flavonoids
1.	Immuno-modulation of inflammatory cells	Modulation of secretory mechanisms Modulation of enzyme actions	Decrease in activity of inflammatory cells.
2.	Immuno-modulation of pro-inflammatory enzymes	Inhibition of NO synthase activity Inhibition of arachidonic acid pathway	Decrease in the production of inflammatory mediators, NO, Prostaglandins (PGs) and leucotrienes (LTs)
3.	Immuno-modulation of pro-inflammatory mediators	Modulation of production of cytokines	Decrease in the production of inflammatory cytokines – TNF alpha, interleukins.
4.	Immuno-modulation of pro-inflammatory gene expression	Modulation of signal transduction	Decrease in pro-inflammatory gene transcription
5.	Anti-oxidant activity	Inhibition of ROS formation Inhibition of pro-oxidant enzymes Radical scavenging mechanims	Decrease in lipid peroxidation and free radicals

(v) Immuno-modulation of proinflammatory molecules

Several cytokines in addition to Cyclooxygenase-2 (COX-2) are associated with inflammatory reactions. Cytokines such as Interleukin-6 (IL-6), Interleukin 1 beta (IL-1β) and Tumour Necrosis Factor alpha (TNF α) are significant contributors to chronic inflammatory diseases. Flavonoid genistein was found to prevent IL-6, IL-1 β and TNFα formation in Liposaccharide (LPS) induced blood monocytes of human origin. [72]. Similar studies revealed the inhibitory action of genistein on IL-6 formation in osteoblast cells [77], gastric epithelial cells [78] and macrophages [79]. It was found that both quercetin and luteolin were capable of inhibiting TNFα production by nearly 80%. Reports on macrophage cell lines RAW 264.7 reveled that genistein, qercetin, luteolin and luteolin 7 glucoside prevented IL-6 and TNF-α production in LPS induced systems [80]. Flavonoids quercetin and wogonin decreased the invitro release of TNF-α and IL-1β, IL-6 in LPS induced RAW cell lines [81, 82].

(vi) Immuno-modulation of proinflammatory genes

Recent research has shown that few flavonoids act as immune-modulators of pro-inflammatory genes leading to attenuation process of inflammatory reactions, thus affecting the messenger RNA (mRNA) levels. The main mechanism by which flavonoids act as immune-modulators was found to be due to its effect on the suppression of transcriptional activity. Genistein, apigenin, kaempferol, catechin, myricetin were found to inhibit COX-2 in LPS induced macrophages [83]. Similarly luteolin lowered mRNA and protein levels of pro-inflammatory COX-2 and induced Nitric Oxide Synthase (iNOS) in LPS-induced macrophages [84]. Various flavonoids were found to inhibit NO production [85-89].

(v) Mechanisms leading to immune-suppression of gene expression

Flavonoids were found to modulate Protein Kinases (PKC) and MAPK (Mitogen Activated Protein Kinases), hence preventing DNA-binding capacity of transcription factors like Activating Protein – 1 (AP-1) and Nuclear Factor k B(NF-k B) [90]. The three main MAPKs are c-Jun-N-terminal kinase (JNK), signal regulated kinases 1 and 2 (Erk 1/2) AND p38 [91]. Studies revealed that p38 - MAPK pathway promote many cytokine genes *invitro* that include IL-6, iNOS, IL-10 [92, 93]. Inhibition of MAPKs is an important step in the anti-inflammatory process. Studies by Means *et al*., showed that luteolin and quercetin inhibited LPS induced promotion of p38 and ERK 1/2 pathways hence preventing the release and production of TNFα [94].

Table 5: Summary of *invitro* studies showing immuno-modulation by flavonoids

S. No.	Activity	Flavonoid	Model	Reference
1.	Modulation of NF-k B	Genistein	Pancreatic cell line	[95]
		Morin	Tumour cell line	[96-98]
		Apigenin	Prostate cancer cell line	[99]
2.	Modulation of Protein Kinase C	Quercetin		[100,101]
		Luteolin	Skin tumour cell lines	
		Apigenin		
3.	Modulation of MAPK	Apigenin	Prostate cancer cell line	[102]
		Genistein	Breast cancer cell line	[103]
		Apigenin		
4.	COX-2 inhibition	Tricin	Adenoma cell line	[104]
		Genistein	Human breastcancer cell lines	[105]

CONCLUSIONS AND FUTURE IMPLICATIONS

The studies involving flavonoids is complex due to its heterogeneity of various molecular structures. They form a group of biologically active substances that are present in high amounts in plants and consumed in large parts in our daily diet. Flavonoids are gaining more and more importance because of their usefulness and significant roles that they play inside the human body. Future implications involve exploring more potential properties of flavonoids in the field of immunomodulation, anti-cataract and anti-toxic activities.

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