Published in final edited form as:
Int J Biol Macromol. 2010 June 1; 46(5): 478–486. doi:10.1016/j.ijbiomac.2010.03.009.

Dietary bioflavonoids inhibit *Escherichia coli* ATP synthase in a differential manner

Nagababu Chinnam, **Prasanna K Dadi**, **Shahbaaz A Sabri**, **Mubeen Ahmad**, **M Anaul Kabir**[#], and **Zulfiqar Ahmad**[*]
Department of Biological Sciences, Box 70703, East Tennessee State University, Johnson City, TN 37614

Abstract

The aim of this study was to determine if the dietary benefits of bioflavonoids are linked to the inhibition of ATP synthase. We studied the inhibitory effect of seventeen bioflavonoid compounds on purified F_1 or membrane bound F_1F_O *E. coli* ATP synthase. We found that the extent of inhibition by bioflavonoid compounds was variable. Morin, silymarin, baicalein, silibinin, rimantadin, amantidin, or, epicatechin resulted in complete inhibition. The most potent inhibitors on molar scale were morin (IC_{50} ~0.07mM) > silymarin (IC_{50} ~0.11mM) > baicalein (IC_{50}~0.29mM) > silibinin (IC_{50} ~0.34mM) > rimantadine (IC_{50} ~2.0mM) > amantidin (IC_{50} ~2.5mM) > epicatechin (IC_{50} ~4.0mM). Inhibition by hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, or rutin was partial in the range of 40–60% and inhibition by galangin, daidzein, or luteolin was insignificant. The main skeleton, size, shape, geometry, and position of functional groups on inhibitors played important role in the effective inhibition of ATP synthase. In all cases inhibition was found fully reversible and identical in both F_1F_o membrane preparations isolated purified F_1. ATPase and growth assays suggested that the bioflavonoids compounds used in this study inhibited F_1-ATPase as well as ATP synthesis nearly equally, which signifies a link between the beneficial effects of dietary bioflavonoids and their inhibitory action on ATP synthase.

Keywords

E. coli ATP synthase; F_1F_o-ATP synthase; F_1-ATPase; ATP synthesis; bioflavonoids; biological nanomotor

Introduction

Membrane bound F_1F_o ATP synthase from mitochondria, chloroplast, and bacteria is responsible for ATP production through oxidative phosphorylation or photophosphorylation. This enzyme is structurally identical and highly conserved in different species. In its simplest form in the ~530 kDa *Escherichia coli* F_1F_o ATP synthase contains eight different subunits namely $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10-15}$. F_1 corresponds to $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_o to ab_2c_{10}. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector, whereas proton transport occurs through

*Corresponding author: Zulfiqar Ahmad, Department of Biological Sciences, Box 70703, East Tennessee State University, Johnson City, TN 37614, Phone: 423-439-6931, Fax: 423-439-5958, ahmadz@etsu.edu.
[#]Present address: School of Biotechnology, National Institute of Technology Calicut, Calicut-673601, Kerala, India

the membrane embedded F_o [1–2]. The γ subunit is part of the "rotor" which is composed of γ, ε, and a ring of c subunits. The "stator" is composed of $b_2\delta$. The function of the stator is to prevent co-rotation of catalytic sites as well as the *a* subunit with the rotor [3–4]. Proton gradient-driven clockwise rotation of γ (as viewed from the membrane) leads to ATP synthesis and anticlockwise rotation of γ results from ATP hydrolysis. The mechanism is essentially a rotary motor and in fact it is the smallest known biological nanomotor. Detailed reviews of ATP synthase structure and function may be found in references [5–11].

ATP synthase is implicated directly or indirectly in several human diseases such as Leigh syndrome, ataxia, Batten's diseases, Alzheimer's, angiogenesis, and increased blood pressure etc ([11] and references therein). This enzyme is not only implicated to many disease conditions but is likely to contribute to new therapies for multiple diseases such as, cancer, heart disease, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and tuberculosis that affect both people and animals [12–13]. The presence of ATP synthase on the surfaces of multiple cell types, and its involvement in a number of cellular processes, makes this enzyme an attractive molecular target, in the development of treatments for numerous diseases. A wide range of natural and synthetic products are known to bind and inhibit ATP synthase [11,13–15] and biochemical and structural studies of ATP synthase have so far revealed about ten different inhibitor binding sites. A detailed list of known inhibitors and their actions on ATP synthase in relation to human heath and disease is discussed in reference [11].

Bioflavonoids/polyphenols are a class of plant secondary metabolites. The beneficial effects of many fruits, vegetables, and tea have been attributed to the presence of bioflavonoid compounds in them. Bioflavonoids are known to exhibit antioxidants, chemopreventive, and chemotherapeutic properties [16–20]. They have been shown to have anti-allergic, anti-inflammatory [21], and anti-microbial activity [22–24]. Their mode of action is not clear, but some dietary bioflavonoids are known to block the action of enzymes and other substances that promote the growth of cancer cells by binding to the multiple molecular targets in the body including ATP synthase [11,13,16,25–26]. For example one of the most common dietary polyphenol resveratrol has been shown to have multiple uses, with multiple benefits in humans, including but not limited to increased life span, anticancer/antitumor effects, and antimicrobial activities [26]. Resveratrol was also shown to induce apoptosis via mitochondrial pathways [25,27]. Aziz et al [28] demonstrated the chemopreventive properties of resveratrol against prostate cancer. They found that treatment with resveratrol concentrations of up to 50μmol/L/day resulted in stimulation of apoptosis in androgen-responsive human prostate carcinoma cells (LNCaP). At similar concentrations resveratrol had no effect on the rate of cell death in normal human prostate cells.

Earlier Zheng and Ramirez [15] studied the inhibitory effects of several naturally occurring polyphenolic phytochemicals on rat brain and liver mitochondrial F_1F_o ATP synthase. They demonstrated that ATP synthase is molecular target for resveratrol and other aglycone isoflavones. Lately, the polyphenols resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3-β-D glucoside, were shown to prevent synthetic or hydrolytic activities of *E. coli* and bovine mitochondrial ATP synthase [13,16]. The proposed mode of action was binding of polyphenols at the polyphenol binding pocket of ATP synthase and blockage of clockwise or anti-clockwise rotation of the γ-subunit [16] (see Figure 1).

The question arises (i) whether the dietary bioflavonoids have differential inhibitory actions on *E. coli* ATP synthase and (ii) what kind of effect dietary bioflavonoids have on the intact *E. coli* cell growth which will be an indicate their effect on ATP synthesis. Thus we studied the inhibitory effect of seventeen bioflavonoid/polyphenol compounds illustrated in Figure 2 on *E. coli* ATP synthase using both purified F_1-ATPase and membrane bound F_1F_o ATP synthase preparations. This study shows that dietary bioflavonoids bind and inhibit *E. coli* ATP

synthase in differential manner. Our results also reaffirm that the beneficial effect of dietary polyphenols as antitumor or antimicrobial agents may be at least in part are through their inhibitory action on ATP synthase.

Materials and Methods

Source of bioflavonoids and other chemicals

Ultra pure bioflavonoid compounds were purchased from Sigma-Aldrich Chemical Company. Catalog numbers for all bioflavonoids used in this study are presented in Table 1. Silymarin used in this study was a mixture of anti-hepatotoxic flavonolignans from the fruit of *Silybum marianum* while silibinin, a pure compound, is the principal component of silymarin. Also, we followed the supplier's directions in the handing of all compounds such as kaempferol was light sensitive so it was protected from light. All the compounds were resuspended in DMSO immediately before use for the desired concentration and were stored in −20 °C. In ATPase assays the final volume of DMSO was not more that 25%. Earlier we noted that up to 40% DMSO has no effect on membrane bound F_1F_o of *E. coli* ATP synthase [13]. All other chemicals used in this study were ultra pure analytical grade, and purchased from either Sigma –Aldrich Chemical Company or Fisher Scientific Company.

Measurement of growth yield in limiting glucose medium; preparation of E. coli membranes; purification of E. coli F_1; assay of ATPase activity of membrane bound F_1F_o or purified F_1

Bothe membrane bound F_1F_o and purified F_1 were isolated from the E. *coli* strain pBWU13.4/DK8 [29]. Growth yield in limiting glucose was measured as in [30]. *E. coli* membrane bound F_1F_o or purified F_1 were prepared as in [31]. It should be noted that this procedure involves three washes of the initial membrane pellets. The first wash is performed in buffer containing 50 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, and 5 mM p-aminobenzamidine. The following two washes are performed in buffer containing 5 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM DTT, and 0.5 mM EDTA. Prior to experiments, membranes were washed twice more by resuspension and ultracentrifugation in 50 mM $TrisSO_4$ pH 8.0, 2.5 mM $MgSO_4$. F_1 was purified as described in Ref [32]. Prior to the experiments, F_1 samples (100μl) were twice passed through 1-ml centrifuge columns (Sephadex G-50) equilibrated in 50mM $TrisSO_4$ pH 8.0, to remove catalytic site bound-nucleotide. ATPase activity was measured in 1 ml of assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, and 50 mM $TrisSO_4$, at pH 8.5 and 37 °C. Reactions were started by addition of 1 ml of assay buffer to the purified F_1 or membranes, and stopped by addition of SDS to a 3.3% final concentration. Pi released was assayed as in [33]. For membrane bound F_1F_o (30 – 50 μg protein), reaction times were 20–30 min. For purified F_1 (20μg protein), reaction times were 2–5 min. All reactions were shown to be linear with time and protein concentration. SDS-gel electrophoresis on 10% acrylamide gels was as in [34]. Immunoblotting with rabbit polyclonal anti-F_1-α and anti-F_1-β antibodies was as in [35].

Inhibition of ATPase activity by bioflavonoid compounds

Membrane bound F_1F_o or purified F_1 (0.2–1.0 mg/ml) were preincubated with varied concentrations of bioflavonoid compounds for 60 min at room temperature, in 50 mM $TrisSO_4$ pH 8.0. The volume of bioflavonoid compounds added was in the ranged from 0–20μl in a total reaction volume of 550μl. Then 1 ml of ATPase assay buffer was added to measure the enzyme activity. Inhibitory exponential decay curves were generated using SigmaPlot 10.0. The best fit lines and IC_{50} values for the curves were obtained using a single 3 parameter model. The range of absolute specific activity for membrane bound F_1F_o was 20–26 and for purified F_1 was 28–42μmol/min/mg at 37 °C for different preparations. These absolute values were used as 100% bench mark to calculate the relative ATPase activity.

Reversal of purified F_1 or membrane bound enzyme ATPase activity from the inhibition of bioflavonoid compounds

Reversibility experiments were performed by dilution of the membrane enzyme and by passing the inhibited purified F_1 through centrifuge columns. For the measurement of reversibility by dilution, membranes were first reacted with inhibitory concentrations of bioflavonoids for 1 hour at room temperature. These concentrations were used based on the maximal observed inhibition of the ATP synthase (see Figure 3–5). 50 mM $TrisSO_4$, pH 8.0 buffer was then added to bring the concentrations down to non inhibitory levels and incubation continued for 1 additional hour at room temperature before the ATPase assay. Reversibility was also tested by passing the bioflavonoid inhibited purified F_1 enzyme through 1 ml centrifuge columns twice before measuring the ATPase activity. Control samples without bioflavonoids were incubated for the same time periods as the samples with bioflavonoids.

Results

Complete inhibition of ATPase activity of purified F_1 or F_1F_o ATP synthase in membranes by morin, silymarin, baicalein, silibinin, rimantadin, amantidin, or, epicatechin

Polyphenol bound X-ray structure has shown the bioflavonoid/polyphenols bound in the polyphenol binding pocket of ATP synthase. This binding pocket located at the interface of α, β, and γ-subunits [16] (Figure 1). [1] The bound bioflavonoids can form hydrophobic interactions with γGln274 (γLys-260), γThr-277 (γIle-263), βAla-264 (βAla-278), or βVal-265 (βVal-279), and additional non polar interactions with residues γAla-270 (γAla-256), γThr-273 (γThr-259), γGlu-278 (γGlu-264), αGly-282 (αGly-290), or αGlu-284 (αGlu-292) which are within 4Å of the bound compounds (Figure 1). *E. coli* residue numbers are used throughout. Bovine mitochondrial residue numbers are shown in parentheses. Polyphenol binding pocket residues of *E. coli* ATP synthase are identical to the bovine polyphenol binding pocket residues except for two changes, namely γQ274K and γT277I, where Gln is replaced by Lys and Thr is replaced by Ile in bovine. The seventeen bioflavonoids used in this study (Fig. 2, Table 1) are dived into three groups: (I) potent inhibitors (~0% residual activity), (II) partial inhibitors (~40–60% residual activity), and (III) weak inhibitors (~80–100% residual activity).

Figure 3 shows the inhibition of ATPase activity of purified F_1 or membrane bound enzyme in presence of varied concentrations of morin, silymarin, baicalein, silibinin, rimantadin, amantidin, or, (−)- epicatechin.. All seven bioflavonoids caused in complete (~100%) inhibition. On molar scale morin hydrate was the most potent inhibitor. The relative potency was morin (IC_{50} ~0.07mM) > silymarin (IC_{50} ~0.11mM) > *baicalein* (IC_{50} ~0.29mM) > silibinin (IC_{50} ~0.34mM) > rimantadine (IC_{50} ~2.0mM) > amantidin (IC_{50} ~2.5mM) > (−)-epicatechin (IC_{50} ~4.0mM). We consistently found that the F_1 data and the membrane data were the same for these inhibitors. This is in agreement with our previously established interpretation that inhibition of ATPase activity can be assayed using either membrane bound F_1F_o preparations or purified F_1 with equivalent results [13,36–40].

Partial inhibition of ATPase activity of purified F_1 or F_1F_o ATP synthase in membranes by hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, or rutin

Figure 4 shows the inhibitory effect of hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, or rutin. These seven bioflavonoids exert partial inhibition of about 40–60%. As before the F_1 data and the membrane bound F_1F_o data were the same for all inhibitors.

[1] *E. coli* residue numbers used throughout.

Insignificant or no inhibition by galangin, daidzein, or luteolin of ATPase activity of purified F_1 and F_1F_o ATP synthase in membranes

The maximal inhibition in the presence of luteolin was ~20%, daidzein was ~10%, and galangin showed no inhibition at all (Fig 5). Partial or slight inhibition of ATP synthase is not uncommon. In previous studies [7,13,36–43], we have noted several instances where mutant or wild-type ATP synthase were incompletely inhibited by inhibitors like fluoroaluminate, fluoroscandium, sodium azide, NBD-Cl, polyphenols, or amphibian peptides. To ensure that the maximal inhibition with bioflavonoids hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, rutin, *galangin, daidzein, or luteolin* had been reached, we incubated each membrane bound F_1F_o preparation or purified F_1 with hesperidin (9mM), chrysin (9mM), kaempferol (1 mM), diosmin (9mM), apigenin (1.5mM), genistein (2mM), rutin (1 mM), galangin (4mM), daidzein (10mM), or luteolin (2mM) by the maximal inhibitory concentrations, for 1 h as in Figure 3 and 4. This was followed by supplementary pulses of the same inhibitory bioflavonoid concentrations and incubation was continued for an additional hour before ATPase assay. As shown in Figure 6A very little or no additional inhibition occurred, which was consistent with Figure 4 and 5 data. This shows that the inhibition by the above bioflavonoids was maximal, and fully inhibited F_1 or membrane bound enzyme retained residual activity. Although, we used a 1 hour incubation time, it was observed that the maximal inhibition of purified F_1 or membrane bound enzyme was achieved within 15 minutes. Earlier resveratrol was shown to inhibit mitochondrial F1Fo ATP synthase within 1–2 minutes[15].

Reversal of ATPase activity of purified F_1 or membrane bound enzyme from the bioflavonoid inhibition

Here we examined whether the bioflavonoid induced inhibition of ATPase is reversible or not. Reversibility data is shown in Figure 6B. This experiment was carried out in two ways. (i) the purified F_1 or membrane bound enzyme was inhibited with the maximum inhibitory concentrations of bioflavonoids for 1 hr at RT as in Figures 3–5. Samples were then diluted to a non-inhibitory concentration and ATPase activity was measured. (ii) 20 μg of purified F_1 samples were incubated with maximum inhibitory concentrations of bioflavonoids for 1 hr at RT. As before the inhibitory concentrations were determined based on data from figures 3 and 4. Inhibited samples were then passed twice through 1 ml sephadex G50 centrifuge columns and ATPase activity measured. Inhibition by all bioflavonoids was found to be fully reversible.

Inhibition of growth on LB, limiting glucose, and succinate medium in presence of bioflavonoid compounds

Inhibitory effects on ATP synthesis were studied by growing the wild-type *E. coli* strain pBWU13.4/DK8 on succinate plates (a non-fermentable carbon source), or limiting glucose, in the presence or absence of bioflavonoid compounds. The abrogation of growth was in proportion to the inhibition ATPase activity (see Table 1).

Discussion

There is increasing interest in the effects of natural dietary compounds as antimicrobial and antitumor agents. For example, polyphenols like resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3-β-glucoside were shown to bind and inhibit ATP synthase suggesting that the dietary benefits of these polyphenols are in part linked to the inhibition of ATP synthesis in tumor cells, thereby leading to apoptosis [11,13,15–16,44]. Thus, the ultimate goal of this study was to examine if the antimicrobial or anticancer properties of dietary bioflavonoids are possibly associated with the inhibition of ATP synthase. Moreover, results from *E. coli* ATP synthase have added advantage in understanding the antimicrobial effects of dietary bioflavonoid compounds.

Bioflavonoid compounds used in this study resulted in complete, partial, slight, or no inhibition of ATP synthase. This trend can be attributed to the interaction between the bioflavonoid compounds and the polyphenol binding pocket residues. Compound skeleton, size, shape, geometry, and functional group presence or position played important role in binding and inhibition (Figure 1 and 2). For example presence of sugar moieties in hesperidin, diosmin, or rutin might cause sterical hindrance resulting in partial inhibition. Fewer –OH group or the extended positioning of –OH group also resulted in partial or insignificant inhibition. On the other hand multiple –OH groups in morin, baichalein, epicatechin, silymarin, or silibinin and presence of NH_2 groups in rimantadine or amantadin resulted in complete inhibition of ATPase activity.

Importance of position and number of –OH groups is also apparent from the differential inhibitory effects of quercetin and apigenin. Both are reversible inhibitors of *E. coli* ATP synthase and bind non-covalently at the polyphenol binding site. Quercetin was shown to induce ~80% inhibition with IC_{50}~33μM [13] while apigenin resulted in partial inhibition ~38% (see Figure 4). Higher affinity and potent inhibition by quercetin (IC_{50} ~55–65μM) in comparison to apigenin (IC_{50} ~100μM) was also observed in the inhibition of mitochondrial F_1F_o ATP synthase [15]. This enhanced inhibitory effect of quercetin can be attributed to the two additional hydroxyl groups on its flavone skeleton, facilitating its inhibitory and binding activity to ATP synthase [13,15,45].

Earlier [15] comparative inhibitory effects of several naturally occurring polyphenolic phytochemicals on rat brain and liver mitochondrial F_1F_o ATP synthase also indicated the importance of hydroxyl groups in a particular position. It was found that genistein, biochanin A, and daidzein all abrogated the ATPase activity with IC_{50} values between ~55 – 127 but genistin a 7-glucose derivative of genistein did not inhibit ATPase activity up to 140 μM. Interestingly enough the inhibitory effects dietary bioflavonoids on mitochondrial F_1F_o did not yield big differences in the degree of inhibition while similar set of bioflavonoids in this study inhibited in very differential manner. We found very different extents of maximal inhibition (from zero to near 100%) as well as very widely different IC_{50} values (from 0.07 mM for morin to 4 mM (−)-epicatechin), representing several logs difference for only subtle modification of the chemical scaffold. Inhibitory effects of the similar set of polyphenolic compounds on mitochondrial enzyme resulted in IC_{50} values between ~50–125μM [15]. This difference between *E. coli* and mitochondrial enzyme results may be due to the fact that Zheng and Ramirez [15] employed a coupled assay to determine ATPase effects (and a direct Pi quantitation for only a few), whereas we measured Pi directly without intervening coupling enzymes. It should be noted that our methods would not be subject to this potentially indirect inhibition- i.e. if the coupling enzymes were inhibited, then this would be masked as a lower activity or false inhibition. Alternatively, it is possible that subtle differences in the *E. coli* enzyme are responsible- allowing a broader range of effects. Also, the use of *E. coli* enzyme seems more appropriate for correlation with antibacterial activities.

Based on the resveratrol, piceatannol, or quercetin bound x-ray structure of the polyphenol binding pocket residues γQ274, γT277, βV265, and βA264 seem to play a critical role in forming hydrophobic interactions with the polyphenol compounds. Other proximal residues which could form non-polar interactions with the bioflavonoid compounds are γA270, γT273, γE278, αE284, αG282, and αE284. The polyphenol binding pocket residues are highly conserved among different species including human, bovine, rat, and *E. coli* [46–47]. However, the *E. coli* enzyme residues γGln-274 and γThr-277 in the polyphenol binding pocket are replaced by γLys-274 and γIle-277 in mitochondrial enzyme. Distance measurements using Deep View Swiss-Pdb Viewer, Version 4.01 (http://spdbv.vital-it.ch/) suggests that the –OH group of γThr-277 generates an additional H-bond with the –OH group of γSer-281 [13] and may form additional H-bonds with the oxygen or –OH groups of the bioflavonoid compounds.

One important aspect of the inhibitory mechanism of bioflavonoids is their effect on rotary mechanism of the enzyme. [2] Mutagenic analysis of polyphenol binding pocket residues demonstrate that the hydrophobic binding pocket between the γ-subunit c-terminal tip and the hydrophobic inside provided by the α- or β-subunit residues is essential for the binding of polyphenol/bioflavonoid compounds.

Addition of extra pulse of compounds to the partially inhibited purified F_1 or membrane bound F_1F_o did not change the level of inhibition significantly (figure 5A). This suggests that the purified F_1 or membrane bound enzyme was fully inhibited by the compounds and the observed extent of inhibition was accurate. Also the partial inhibition is not a result of uninhibited enzyme or degradation of the compounds with time. The process of inhibition was also found to be completely reversible. A fully reacted F_1 regained activity after being passed through the centrifuge columns to remove the compounds. Similarly, purified F_1 or membrane bound enzyme regained activity following exposure to higher concentrations, once returned to lower concentrations of the inhibitory compound, by dilution with buffer.

The growth pattern of *E. coli* in the presence of seventeen bioflavonoid compounds is presented in Table 1. Remarkably, the loss of growth and ATPase inhibition in the presence of all the compounds used in this study was nearly equivalent. The pUC118/DK8 (null strain) usually grows from 40–50% of the pBWU13.4/DK8 (wild-type, Table 1). This is because the null strain uses only glycolysis to generate ATP, whereas the wild-type uses both glyolysis and oxidative phosphorylation. Up to 40–50% retention of growth in the presence of each of bioflavonoid compounds is the result of intact substrate level phosphorylation, suggesting that loss of growth (50–60%) is caused by abrogation of oxidative phosphorylation resulting from inhibition of ATP synthesis. These results support the initial hypothesis that some of the beneficial effects of dietary bioflavonoids may be attributable to their inhibitory effects on ATP synthase. However, in earlier studies the degree of inhibition of F_1-ATPase and ATP synthesis in presence of some polyphenols, peptides, or antibiotic was also found to be different [13–14,48].

The uncontrolled growth of tumor cells requires additional energy in the form of ATP generated by ATP synthase. Thus the inhibition of ATP synthase will deprive tumor cells of required energy and lead to cell death or apoptosis [13,16]. For example, resveratrol was shown to induce apoptosis via a mitochondrial pathway [25,27]. Oligomycin, another specific ATP synthase inhibitor induced an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells within 12–18 hrs, but not in ρ° cells that are depleted of a functional mitochondrial respiratory chain [49]. Thus inhibition of the components of mitochondrial pathways may lead to marking of some cells, via CD14, for cell death, while allowing commitment to differentiation to occur in the surviving population [50]. Cell death can also be prompted through alteration of cellar bioenergetics. 1, 4-benzodiazepine (Bz-423) was shown to bind and inhibit the oligomycin sensitivity – conferring protein subunit of F_1F_o ATP synthase. This results in significant decrease in ATP synthesis and increase in the production of free radicals which in turn activates redox regulated apoptosis [51].

Polyphenol induced inhibition of proton-translocating F_1-ATPase activity of *S. mutans* resulting in inhibition of biofilm formation and acid production [22,52], and inhibition of ATPase activity as well as intact *E. coli* cell growth indicated that ATP synthase is a potential molecular target for dietary bioflavonoids. Two c-subunit mutations, D32V and A63P, of mycobacterium synthase confer resistance against the anti-tuberculosis drug diarylquinoline [53–54]. This also supports earlier conclusions that potent ATP synthase inhibitors may

[2] *E. coli* enzyme mutants namely αR283A/Q, αE284A/Q, βS263A/Q, γT273A/Q, γQ274A/K, γT277A/Q, and γE278A/Q resulted in the loss of inhibition by resveratrol, piceatannol and quercetin. (Dadi, P.K., and Ahmad, Z. unpublished results)

stimulate the development of specific antibacterial drugs, and drugs that target tumor cells without affecting normal cells [13,16].

Acknowledgments

This work was supported by the National Institutes of Health [Grant GM085771] and East Tennessee State University Small RDC [Grant 211457] to ZA.

Abbreviations used

NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole
Mbr	membrane containing ATP synthase
IC_{50}	corresponds to the concentration of inhibitor where 50% of maximal inhibition was observed.

References

1. Senior AE, Nadanaciva S, Weber J. Biochim Biophys Acta 2002;1553:188–211. [PubMed: 11997128]
2. Abrahams JP, Leslie AGW, Lutter R, Walker JE. Nature 1994;370:621–628. [PubMed: 8065448]
3. Diez M, Zimmermann B, Borsch M, Konig M, Schweinberger E, Steigmiller S, Reuter R, Felekyan S, Kudryavtsev V, Seidel CAM, Graber P. Nat Struct Mol Biol 2004;11:135–141. [PubMed: 14730350]
4. Itoh H, Takahashi A, Adachi K, Noji H, Yasuda R, Yoshida M, Kinosita K. Nature 2004;427:465–468. [PubMed: 14749837]
5. Senior AE. Cell 2007;130:220–221. [PubMed: 17662937]
6. Weber J, Senior AE. FEBS Lett 2003;545:61–70. [PubMed: 12788493]
7. Ahmad Z, Senior AE. J Bioenerg Biomembr 2005;37:437–440. [PubMed: 16691479]
8. Frasch WD. Biochim Biophys Acta 2000;1458:310–325. [PubMed: 10838047]
9. Ren H, Allison WS. Biochim Biophys Acta 2000;1458:221–233. [PubMed: 10838039]
10. Noji H, Yoshida M. J Biol Chem 2001;276:1665–1668. [PubMed: 11080505]
11. Hong S, Pedersen PL. Microbiol Mol Biol Rev 2008;72:590–641. [PubMed: 19052322]
12. Pedersen PL. J Bioenerg Biomembr 2007;39:1–12. [PubMed: 17404823]
13. Dadi PK, Ahmad M, Ahmad Z. Int J Biol Macromol 2009;45:72–79. [PubMed: 19375450]
14. Thorsen F, Enger PO, Wang J, Bjerkvig R, Pedersen PH. J Neurooncol 2007;82:1–10. [PubMed: 16955221]
15. Zheng J, Ramirez VD. Br J Pharmacol 2000;130:1115–1123. [PubMed: 10882397]
16. Gledhill JR, Montgomery MG, Leslie AG, Walker JE. Proc Natl Acad Sci U S A 2007;104:13632–13637. [PubMed: 17698806]
17. Barta I, Smerak P, Polivkova Z, Sestakova H, Langova M, Turek B, Bartova J. Neoplasma 2006;53:19–25. [PubMed: 16416008]
18. Nishino H, Murakoshi M, Mou XY, Wada S, Masuda M, Ohsaka Y, Satomi Y, Jinno K. Oncology 2005;69:38–40. [PubMed: 16210876]
19. Athar M, Back JH, Kopelovich L, Bickers DR, Kim AL. Arch Biochem Biophys 2009;486:95–102. [PubMed: 19514131]
20. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, Kim AL. Toxicol Appl Pharmacol 2007;224:274–283. [PubMed: 17306316]
21. Yamamoto Y, Gaynor RB. J Clin Invest 2001;107:135–142. [PubMed: 11160126]
22. Percival RS, Devine DA, Duggal MS, Chartron S, Marsh PD. Eur J Oral Sci 2006;114:343–348. [PubMed: 16911106]
23. Cushnie TP, Lamb AJ. Int J Antimicrob Agents 2005;26:343–356. [PubMed: 16323269]

24. Duarte AR, Gordillo MD, Cardoso MM, Simplicio AL, Duarte CM. Int J Pharm 2006;311:50–54. [PubMed: 16423476]
25. Pervaiz S. FASEB J 2003;17:1975–1985. [PubMed: 14597667]
26. Pirola L, Frojdo S. IUBMB Life 2008;60:323–332. [PubMed: 18421779]
27. Clement MV, Hirpara JL, Chawdhury SH, Pervaiz S. Blood 1998;92:996–1002. [PubMed: 9680369]
28. Aziz MH, Nihal M, Fu VX, Jarrard DF, Ahmad N. Mol Cancer Ther 2006;5:1335–1341. [PubMed: 16731767]
29. Ketchum CJ, Al-Shawi MK, Nakamoto RK. Biochem J 1998;330:707–712. [PubMed: 9480879]
30. Senior AE, Latchney LR, Ferguson AM, Wise JG. Arch Biochem Biophys 1984;228:49–53. [PubMed: 6230049]
31. Senior AE, Langman L, Cox GB, Gibson F. Biochem J 1983;210:395–403. [PubMed: 6222731]
32. Weber J, Lee RS, Grell E, Wise JG, Senior AE. J Biol Chem 1992;267:1712–1718. [PubMed: 1530942]
33. Taussky HH, Shorr E. J Biol Chem 1953;202:675–685. [PubMed: 13061491]
34. Laemmli UK. Nature 1970;227:680–685. [PubMed: 5432063]
35. Rao R, Perlin DS, Senior AE. Arch Biochem Biophys 1987;255:309–315. [PubMed: 2884928]
36. Ahmad Z, Senior AE. J Biol Chem 2004;279:31505–31513. [PubMed: 15150266]
37. Ahmad Z, Senior AE. J Biol Chem 2005;280:27981–27989. [PubMed: 15939739]
38. Brudecki LE, Grindstaff JJ, Ahmad Z. Arch Biochem Biophys 2008;471:168–175. [PubMed: 18242162]
39. Li W, Brudecki LE, Senior AE, Ahmad Z. J Biol Chem 2009;284:10747–10754. [PubMed: 19240022]
40. Laughlin TF, Ahmad Z. Int J Biol Macromol 2010;46:367–374. [PubMed: 20100509]
41. Ahmad Z, Senior AE. J Biol Chem 2004;279:46057–46064. [PubMed: 15322126]
42. Ahmad Z, Senior AE. FEBS Lett 2005;579:523–528. [PubMed: 15642370]
43. Ahmad Z, Senior AE. FEBS Lett 2006;580:517–520. [PubMed: 16405964]
44. Zheng J, Ramirez VD. Biochem Biophys Res Commun 1999;261:499–503. [PubMed: 10425214]
45. Wu D, Kong Y, Han C, Chen J, Hu L, Jiang H, Shen X. Int J Antimicrob Agents 2008;32:421–426. [PubMed: 18774266]
46. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, Williams RL. Mol Cell 2000;6:909–919. [PubMed: 11090628]
47. Sicheri F, Moarefi I, Kuriyan J. Nature 1997;385:602–609. [PubMed: 9024658]
48. Perlin DS, Latchney LR, Senior AE. Biochim Biophys Acta 1985;807:238–244. [PubMed: 2859888]
49. Wolvetang EJ, Johnson KL, Krauer K, Ralph SJ, Linnane AW. FEBS Lett 1994;339:40–44. [PubMed: 8313978]
50. Mills KI, Woodgate LJ, Gilkes AF, Walsh V, Sweeney MC, Brown G, Burnett AK. Biochem Biophys Res Commun 1999;263:294–300. [PubMed: 10491287]
51. Johnson KM, Cleary J, Fierke CA, Opipari AW Jr, Glick GD. ACS Chem Biol 2006;1:304–308. [PubMed: 17163759]
52. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H. FEMS Microbiol Lett 2006;257:50–56. [PubMed: 16553831]
53. Andries K, Verhasselt P, Guillemont J, Gohlmann HWH, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. Science 2005;307:223–227. [PubMed: 15591164]
54. Cole ST, Alzari PM. Science 2005;307:214–215. [PubMed: 15653490]
55. Sayle RA, Milner-White EJ. Trends Biochem Sci 1995;20:374. [PubMed: 7482707]

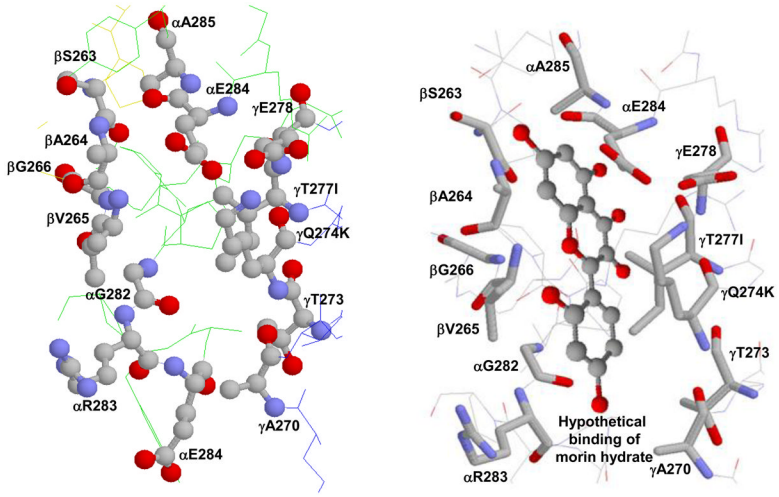

Figure 1.

X-ray crystallographic structure of polyphenol binding site of ATP synthase. (A) Empty and (B) hypothetical binding of morin hydrate at the polyphenol binding pocket. Residues from α, β, and γ subunits involved in interaction with polyphenols are identified. In bovine two variants, Q274K and T277I, occur in the γ subunit and are identified in the figure. PDB file 2jj1 [16] with RasMol [55] was used to generate this figure.

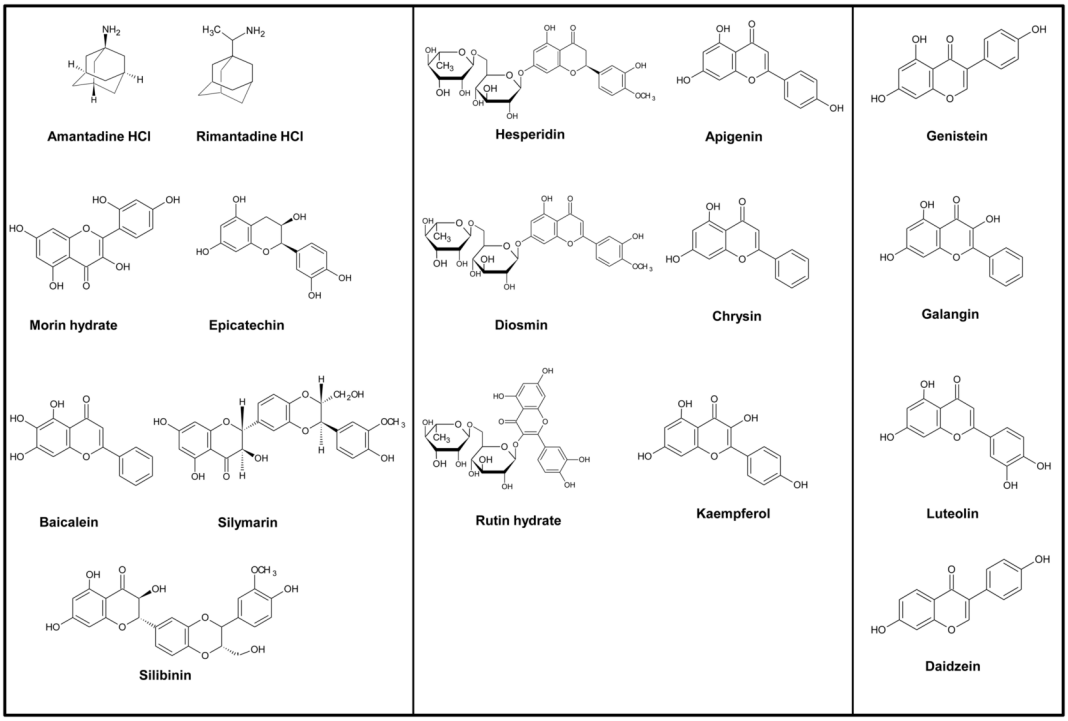

Figure 2. Structures of bioflavonoids are shown in three groups

(I) exerting complete inhibition of F_1-ATPase activity, (II) exerting incomplete inhibition of F_1-ATPase activity, and (III) exerting insignificant inhibition of F_1-ATPase activity.

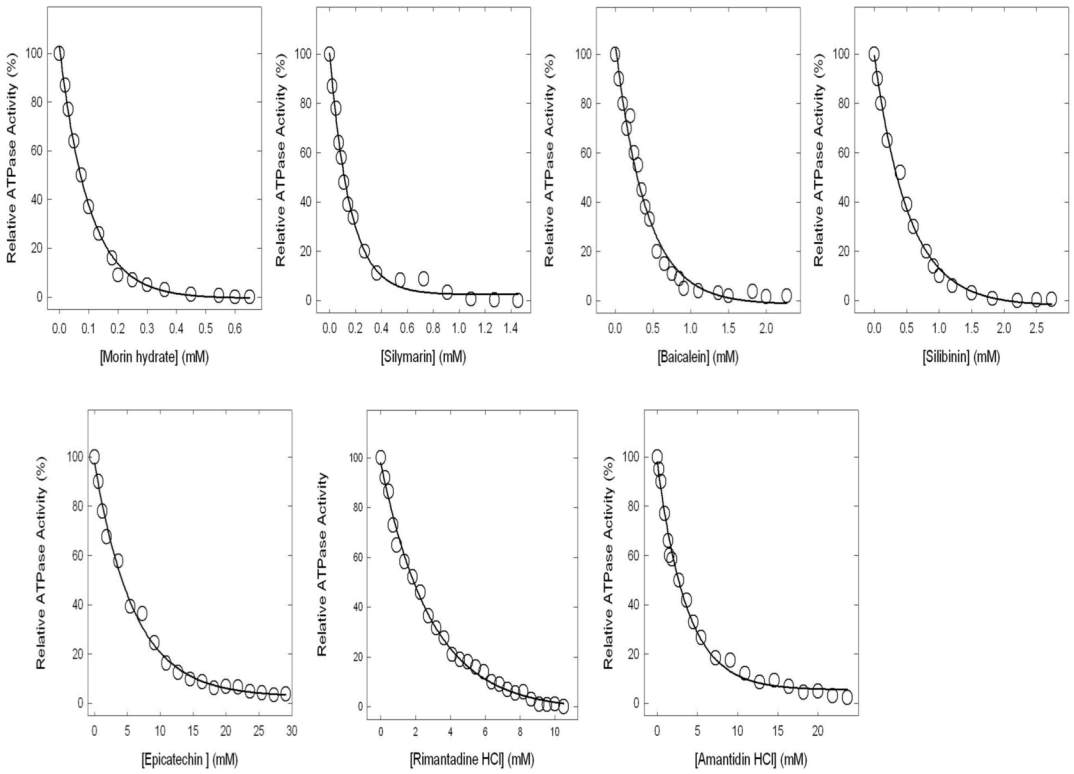

Figure 3. Complete inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase

Membranes or purified F_1 were preincubated for 60 min at 23°C with varied concentration of bioflavonoids shown in the figure and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details are given in Materials and Methods. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within ± 10%.

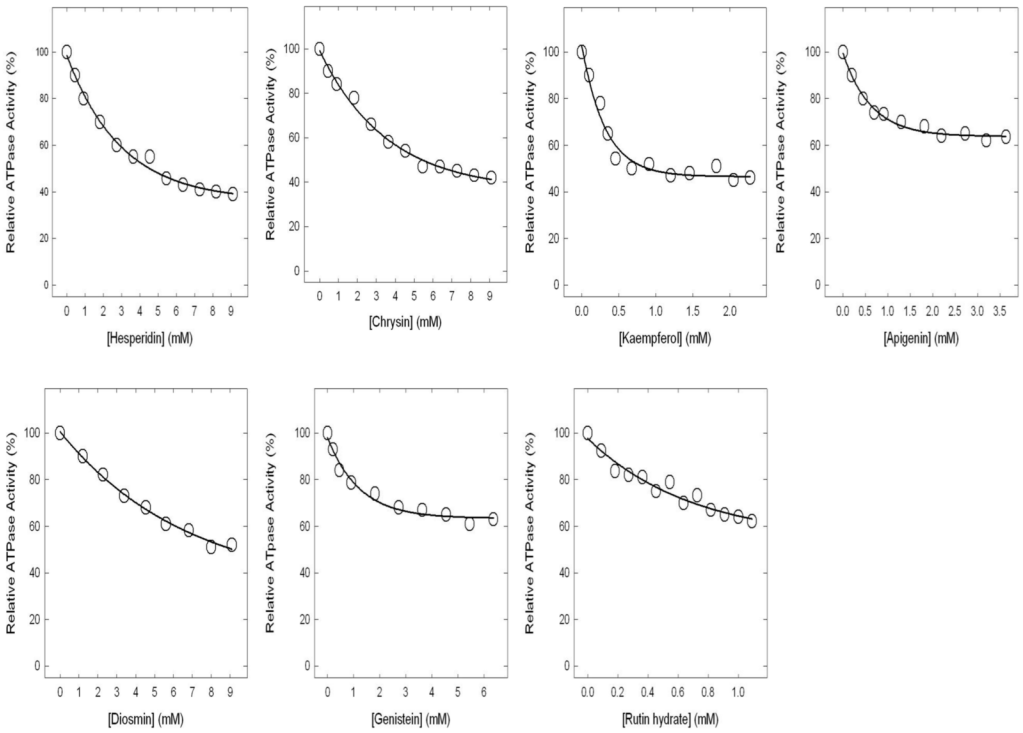

Figure 4. Incomplete inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase

Membranes or purified F_1 were preincubated for 60 min at 23°C with varied concentration of bioflavonoids identified in the figure and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details can be found Materials and Methods section. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within ± 10%.

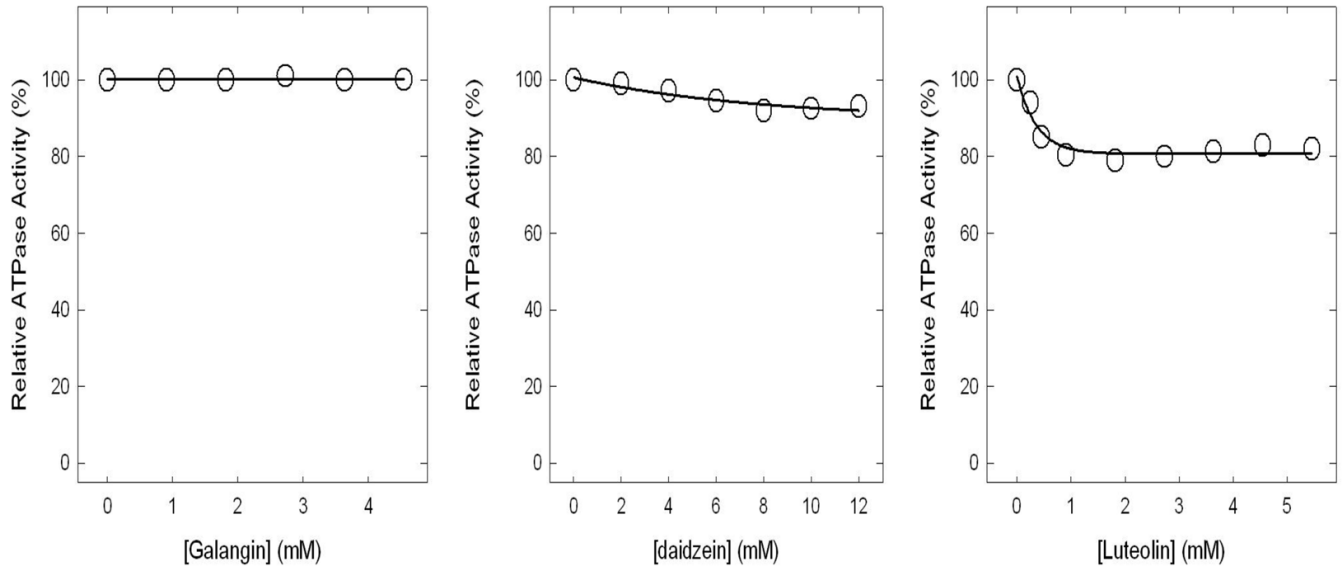

Figure 5. Insignificant or no inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase

Membranes or purified F_1 were preincubated for 60 min at 23°C with varied concentration of bioflavonoids identified in the figure and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within ± 10%.

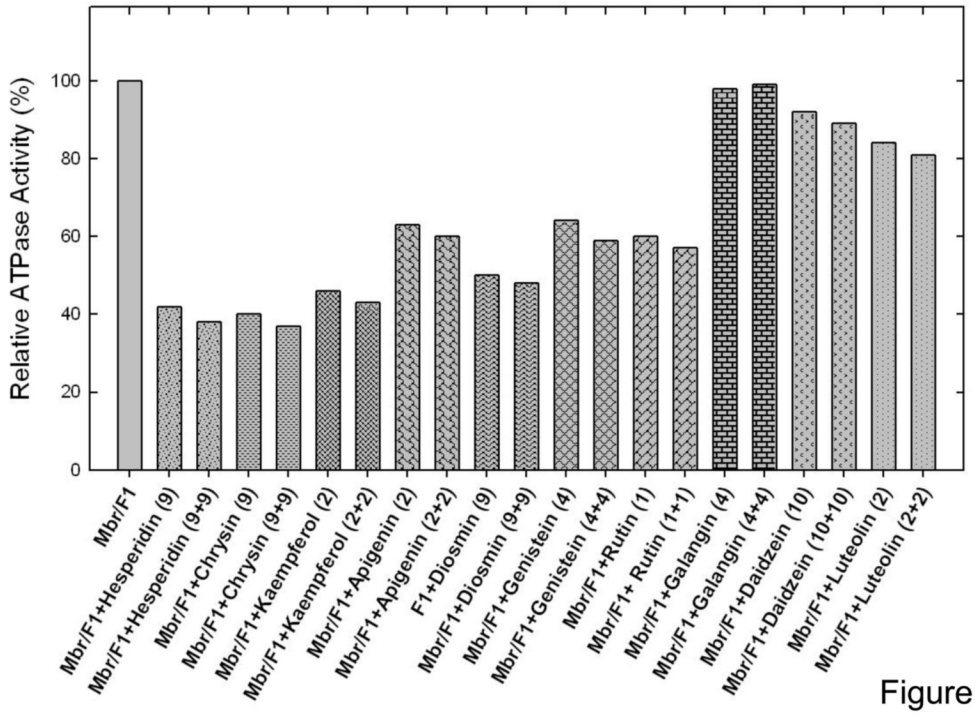

Figure 6A

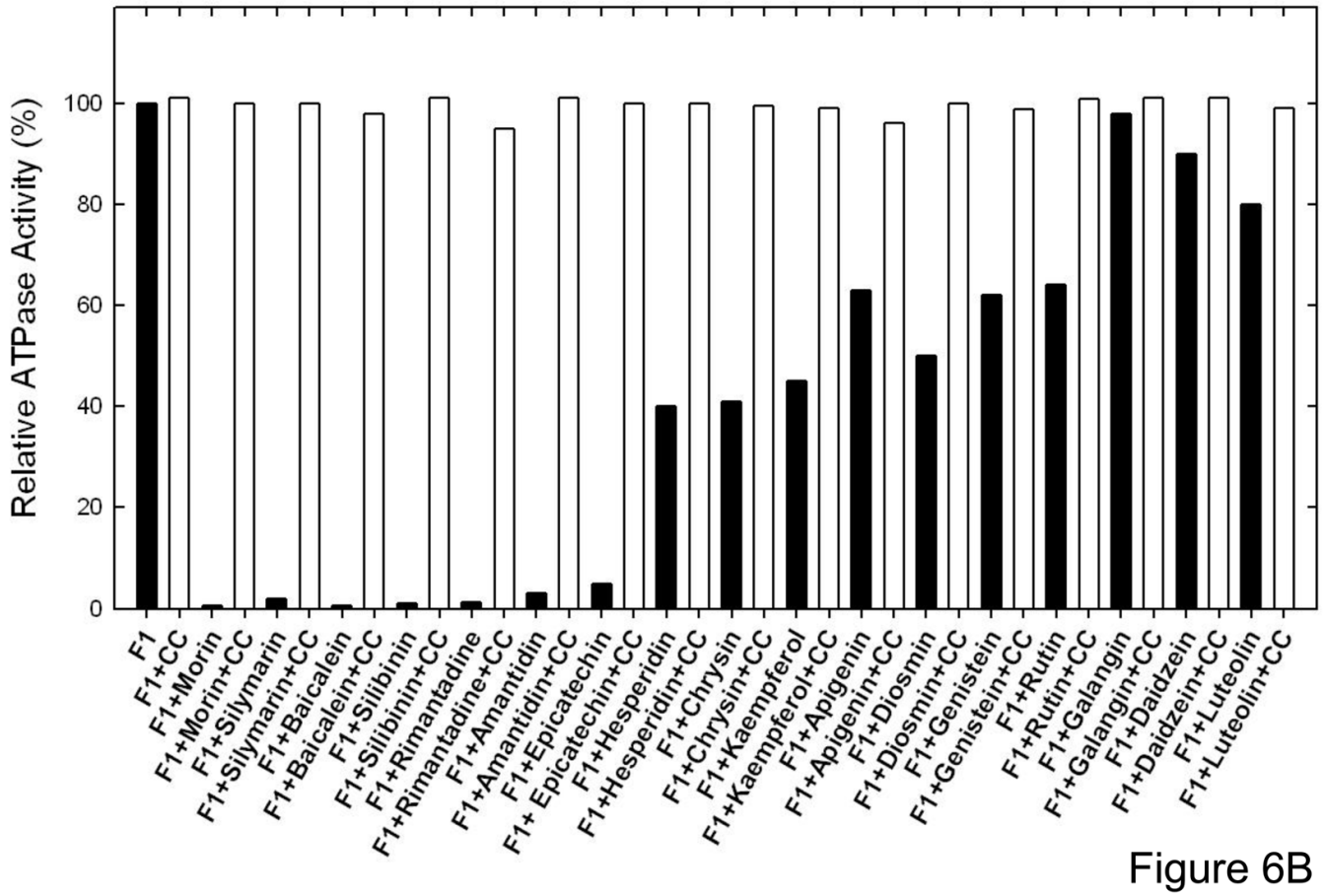

Figure 6B

Figure 6. Results of Extra pulses of bioflavonoid compounds and reversal of inhibition by passing through centrifuge columns

(A), Membrane bound ATP synthase (Mbr) or purified F_1 (F_1) was inhibited with inhibitory concentrations of the bioflavonoid compounds shown in the figure for 60 min under conditions as described in Fig 3–4. Then a further pulse of identical inhibitory concentrations was added and incubation continued for 1 h before assay. The last digits represent the compound concentrations in [μM]. (B) Purified F_1 was incubated with inhibitory concentrations of bioflavonoid compounds for 60 min under conditions as described in Fig 2–4. Then the inhibited samples were passed twice through 1 ml centrifuge columns and ATPase activity was measured. The first bar is for purified F_1 with no compound (F1), followed by bars in presence of compounds.

Table 1

Growth of *Escherichia coli* cells and residual ATPase activity in presence of bioflavonoid compounds

Polyphenolic compounds	Sigma catalog numbers	[a] Growth on succinate plates	[b] Growth yield in limiting glucose (%)	[c] Residual ATPase Activity (%)	IC50 values (mM)
[d] Control	N/A	++++	100	N/A	N/A
[e] Null	N/A	-	45	N/A	N/A
Morin hydrate	M4008-5G	-	46	0	0.07
Silymarin	S0292-10G	-	51	0	0.11
Baicalein	465119-100MG	-	48	0	0.29
Silibinin	S0417-1G	-	47	0	0.34
Rimantadine	390593-1G	-	52	0	2.0
Amantadin	A1260-5G	-	51	0	2.5
(−)-Epicatechin	E1753-1G	-	53	0	4.0
Hesperidin	H5254-25G	++	76	40	N/A
Chrysin	C80105-25MG	++	73	40	N/A
Kaempferol	K0133-10MG	++	74	45	N/A
Diosmin	D3525-5G	+++	84	50	N/A
Apigenin	A3145-25MG	+++	84	60	N/A
Genistein	G6649-25MG	+++	87	62	N/A
Rutin hydrate	R5143-50G	+++	87	60	N/A
Galangin	282200-25MG	++++	93	100	N/A
Daidzein	D7802-25MG	++++	96	90	N/A
Luteolin	L9283-10MG	++++	87	80	N/A

[a]Growth on succinate plates after 3 days was determined by visual inspection. ++++, high growth; +++, moderate growth; ++, low growth; or -, no growth.

[b]Growth yield on limiting glucose was measured as OD_{595} after ~20 hours growth at 37 °C.

[c]Residual activity is the left over ATPase activity

[d, e]Control, pBWU13.4/DK8 which contains UNC^{+} gene encoding ATP synthase; Null, pUC118/DK8 with UNC^{-} gene. Growth of positive and negative controls in absence of polyphenol compounds. Data are means of four to six experiments each at 37 °C. Each individual experimental point is itself the mean of duplicate assays.