Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. (Oct–Dec 2022) 92(4):939–946
https://doi.org/10.1007/s40011-022-01396-6

RESEARCH ARTICLE

Effect of Different Proportions of Phenolics on Antioxidant Potential: Pointers for Bioactive Synergy/Antagonism in Foods and Nutraceuticals

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Received: 17 December 2021 / Revised: 17 December 2021 / Accepted: 10 May 2022 / Published online: 31 July 2022

Abstract Phenolic compounds include a broad variety of antioxidant plant substances such as flavonoids that have in common an aromatic ring with one or more hydroxyl groups. Nutraceuticals and health food supplements are designed from flavonoids as well as pure phytochemicals, often in isolation. However, studies on synergistic and antagonistic effects of such compounds are relatively few. In the current study, dual combinations prepared from five phenolic compounds (flavonoid and non-flavonoid) including rutin hydrate, quercetin dihydrate, hydroquinone, kaempferol, and resveratrol were tested for their antioxidant activities using $DPPH^{\cdot}$ radical scavenging assay. The synergistic antioxidant interactions among these phenolics were evaluated by comparing their individual antioxidant effect with that obtained by a mixture of two compounds in various ratios. Quercetin dihydrate showed the highest antioxidant activity. Many combinations were found statistically synergistic in particular ratios. Rutin hydrate and resveratrol showed maximum synergy (1:1, 2:1, and 3:1 ratio). Antagonistic interactions were also identified. The results of this study could be used by industries to develop more potent nutraceutical supplements or guide the researchers for further bioactivity validation using in vivo assays.

Keywords Phenolics · Flavonoids · Antioxidant activity · DPPH assay · Synergy · Antagonism

Introduction

Phytochemicals have long been the most plentiful source of health care and life enhancement for humans. In addition to medicinal applications, they have been used in cosmetics, health, aroma, and as dietary supplements. The market for phytochemical nutraceuticals has continuously increased over the past few years (USD 353 billion in 2019) with many new companies entering the market. The recent global pandemic COVID-19 has further led to a surge in people opting for immune-boosting dietary supplements and nutraceuticals [1]. Also, due to their presence in the fruits and vegetables we eat, it is easier to incorporate them into our diet [2]. Phenolic compounds include a broad variety of plant substances that have in common an aromatic ring with one or more hydroxyl groups. Flavonoids are the most abundant phenolics and are divided into several subgroups: flavanols, flavanones, flavonols, flavones, anthocyanidins, and isoflavones based on the substitution and oxidation levels of the A, B, and C rings of the flavan nucleus which is the basic flavonoid structure (Fig. 1). These structural features such as the ortho-dihydroxy structure in the B-ring, the 2–3-double bond in conjugation with a 4-oxo function, and the presence of the 3- and 5-OH functions are thought to be closely linked to a compound's antioxidant potency [3]. Interestingly, the flavonoids can interact with each other which can impact the total antioxidant capability [4]. In fact, the interactions between

Significance statement: Here, we have tested synergy and antagonism among phytochemicals. The results of this study could be used by industries to develop more potent nutraceutical supplements or guide the researchers for further bioactivity validation using in vivo assays.

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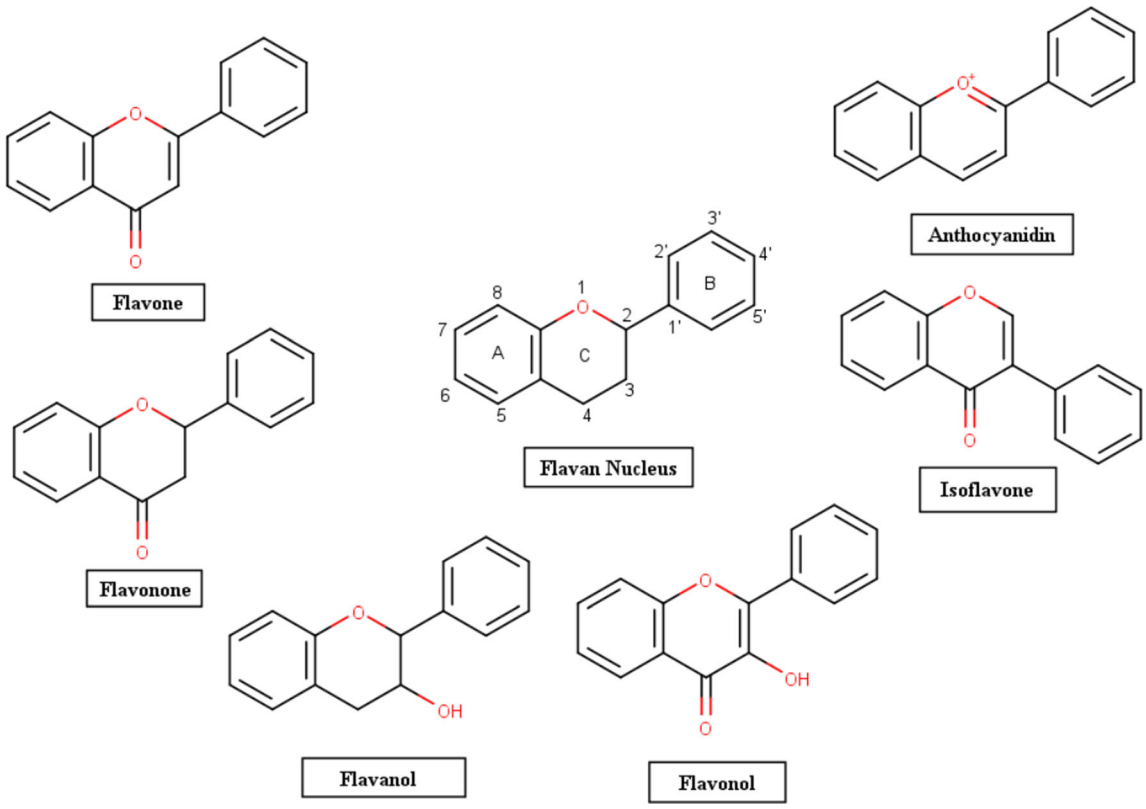

Fig. 1 Basic flavan structure and chemical structures of flavonoid subgroups. Structures are drawn using Marvin sketch software

phytochemicals can be additive, synergistic, or antagonistic. The antagonistic effects of phytochemicals may be exacerbated by excessive doses, inappropriate use, or drug interactions [5].

Food synergy is a concept that describes the positive or negative interactions between nutrients, their absorption, and bioavailability in the human body [6]. It is characterized by effects due to the interaction of nutrients in a variety of foods or whole foods rather than a single food component. For instance, green tea and black pepper have a synergistic effect, on account of increasing the bioavailability of epigallocatechin gallate (EGCG), a compound found in green tea [2]. Similarly, the polyherbal combination of *Vitis vinifera*, *Phyllanthus emblica* L., *Punica granatum, Cinnamomum cassia,* and *Ginkgo biloba* L. with green tea was found to show the highest antioxidant activity as compared to the individual extracts [7]. In an important study, Shoba et al. [8] had shown that at certain dosages, piperine enhanced the bioavailability of curcumin in both animal and human volunteers; the results of this study are used today by food companies to develop ready-to-eat items such as instant beverages (e.g. MTR rasam). In another study, it was found that individually, red grape and carrot had the lowest radical scavenging activity (68 ± 1.81 and $33 \pm 5.79\%$, respectively), but when they were combined, the antioxidant potential improved significantly to $93 \pm 0.91\%$ [9]. Although such studies have been restricted in nature, they seem to highlight that if molecules require pairing for functioning, eating them together in the same food increases the chances of pairing. Alternatively, eating various foods during the same 24-h period may be sufficient for pairing to occur within the digestive tract or systemically [10].

In the "flavonol" category of flavonoids, quercetin, kaempferol, and rutin hydrate are the most abundant compounds found in plants. A variety of fruits and vegetables are high in quercetin and kaempferol. Quercetin is found in high concentrations in a few foods, such as onion, asparagus, and berries. Green leafy vegetables, such as spinach and kale, are the greatest plant sources of kaempferol [11]. Capers have the greatest concentration of rutin, quercetin (234 mg/100 g), and kaempferol (259 mg/100 g edible part) [12]. Antioxidants such as mangiferin, quercetin, kaempferol and gallic acid are abundant in mango [13]. Resveratrol is a naturally occurring stilbene found in a variety of vegetables. Dark chocolate, peanuts, cranberry, grape peel, and guava by-products are all edible sources of resveratrol [14]. Similarly, seeds of *Prosopis cineraria* [15], the root bark of *Capparis decidua* [16], and seeds of *Cordia dichotoma* [17] contain rutin, luteolin, gallic acid, etc.

While there are numerous studies that test the antioxidant activity of individual compounds, limited research has been done on the interaction between flavonoid and non-flavonoid phenolic compounds. Based on their common occurrence in plant-derived foods, the following flavonoids were chosen for the current study: rutin hydrate, quercetin dihydrate, and kaempferol. The non-flavonoid phenolics studied were: hydroquinone and resveratrol. Here, we have evaluated the antioxidant activity of individual phenolics and the pairwise interaction between them for possible synergistic or antagonistic activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The findings of the current study may aid in the development of synergy-driven functional foods or health food supplements derived from a combination of different ingredients in a particular ratio based on optimum antioxidant activity.

Material and Methods

Chemicals

DPPH was purchased from HiMedia (India). Methanol, rutin hydrate, kaempferol, and hydroquinone were from Sigma Company (United States), quercetin dihydrate from SRL (India), and resveratrol was purchased from TCI (Shanghai, China).

DPPH· Radical Scavenging Assay

The free radical scavenging ability was tested by DPPH assay as per the method described in [18] with some modifications. The methanolic solution of DPPH is purple/violet coloured, which fades to pale yellow in the presence of antioxidants, and the loss in absorbance is measured spectrophotometrically at 517 nm. A 100 µM DPPH solution was prepared in 95% methanol and 290 µl of this solution was mixed with 10 µl of the antioxidant solution prepared in methanol. The concentration of phenolic compounds was kept between 100 and 500 µM and mixed in different ratios (3:1, 2:1, 1:1, 1:2, 1:3). The reaction was carried out in a 96 well microplate, incubated in the dark at room temperature for 1 h, and absorbance was measured at 517 nm by using a microplate reader (ThermoScientific Multiskan Go). The percentage DPPH inhibition (radical scavenging activity) was calculated by the following equation:

$$\text{Inhibition}\% = \frac{A_c - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample. Solution without the sample was taken as control. The results were expressed as EC_{50} (µM) obtained by plotting a curve between concentration and inhibition percentage. EC_{50} is the effective concentration necessary to get 50% inhibition. Lower the EC_{50} value higher will be the antioxidant activity.

Difference in DPPH Antioxidant Activity

The difference in DPPH antioxidant activity (%) was calculated by the following equation- $100 - [\text{Mix}EC_{50} \times 200]/\text{A } EC_{50} + \text{BEC}_{50}$ [4]. Here, Mix EC_{50} is the value of EC_{50} obtained by the mixture of two compounds, A EC_{50} is the EC_{50} of compound A, and B EC_{50} is the EC_{50} of compound B. Positive values indicate synergistic interaction whereas negative values indicate antagonistic interaction.

Statistical Analysis

Experiments were done in triplicate and the values were calculated as mean ± standard deviation. The means were compared using one-way analysis of variance (ANOVA), and the least significant difference (LSD) test was performed to assess statistically significant difference between the various phenolic combination groups. P-value ≤ 0.05, 0.01 and 0.001 was considered statistically significant.

Results and Discussion

Structure plays an important role in determining the metabolism and functional properties of biologically active molecules which we mostly intake through food [19]. In case of flavonoids, the placement of functional groups around the nuclear structure determines the antioxidant action. The hydroxyl group of B ring transfers hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizes them, and gives rise to relatively stable flavonoid radical; therefore, B ring hydroxyl configuration is the most important factor with respect to scavenging of ROS and RNS [20]. In the current study, besides choosing different flavonoids, we have also tested their antioxidant effect in combination with non-flavonoid phenolics, thereby reaching closer to the real picture present in natural foods. The experimental results of individual compounds were compared with the combination of two compounds and evaluated for synergistic or antagonistic interactions. The results are summarized in Table 1.

When the antioxidant activity of individual compounds was analyzed, quercetin dihydrate showed the highest activity. Figure 2 a, b shows that with increasing concentration, quercetin dihyrate shows the highest inhibition % and lowest EC_{50} value, indicating highest antioxidant activity. The radical scavenging ability of phenolic

Table 1 Antioxidant activity of flavonoids and their combinations evaluated by DPPH assay

S.No	Combination of phytochemicals in different ratio		EC_{50} (μM) ± STDEV	Difference in DPPH antioxidant activity (%)	*P*-value
	P1	*P2*			
1	*Rutin hydrate*	*Quercetin dihydrate*			
	1	0	323.65 ± 27.26		
	1	1	305.77 ± 19.26	− 0.67 ± 2.38	a^{-}, b^{-}
	2	1	315.87 ± 3.49	− 4.17 ± 4.59	a^{-}, $b^{\$}$
	3	1	317.49 ± 7.00	− 4.71 ± 5.21	a^{-}, $b^{\#}$
	0	1	283.65 ± 4.82		
	1	2	312.22 ± 7.23	− 2.91 ± 2.91	a^{-}, $b^{\#}$
	1	3	295.29 ± 15.31	2.63 ± 6.31	a^{-}, b^{-}
2	*Rutin hydrate*	*Hydroquinone*			
	1	0	310.78 ± 8.60		
	1	1	414.94 ± 33.22	0.21 ± 6.31	$a^{\#}$, $b^{\#}$
	2	1	383.55 ± 21.51	7.66 ± 5.76	$a^{\#}$, $b^{\$}$
	3	1	383.12 ± 38.25	7.12 ± 7.50	a*, $b^{\#}$
	0	1	520.34 ± 13.34		
	1	2	444.33 ± 40.72	− 6.86 ± 8.22	$a^{\#}$, b*
	1	3	494.46 ± 74.52	− 18.80 ± 15.77	a*, b^{-}
3	*Rutin hydrate*	*Kaempferol*			
	1	0	310.18 ± 2.27		
	1	1	339.70 ± 16.84	1.49 ± 3.83	a*, b*
	2	1	324.83 ± 14.83	5.77 ± 4.38	a^{-}, $b^{\#}$
	3	1	325.38 ± 17.59	5.62 ± 4.82	a^{-}, $b^{\#}$
	0	1	379.31 ± 9.27		
	1	2	344.90 ± 12.83	− 0.03 ± 3.25	$a^{\#}$, $b^{\#}$
	1	3	347.50 ± 13.73	− 0.77 ± 2.86	$a^{\#}$, b*
4	*Rutin hydrate*	*Resveratrol*			
	1	0	297.21 ± 5.74		
	1	1	409.38 ± 20.01	30.56 ± 1.72	$a^{\$}$, $b^{\$}$
	2	1	364.15 ± 25.42	38.27 ± 1.82	$a^{\#}$, $b^{\$}$
	3	1	348.28 ± 23.47	40.92 ± 3.10	a*, $b^{\$}$
	0	1	881.80 ± 52.77		
	1	2	489.71 ± 1.26	16.84 ± 3.47	$a^{\$}$, $b^{\$}$
	1	3	550.32 ± 19.55	6.46 ± 7.03	$a^{\$}$, $b^{\$}$
5	*Quercetin dihydrate*	*Hydroquinone*			
	1	0	269.12 ± 2.78		
	1	1	347.81 ± 7.51	9.56 ± 1.33	$a^{\$}$, $b^{\$}$
	2	1	320.96 ± 14.83	16.43 ± 6.34	$a^{\#}$, $b^{\$}$
	3	1	316.32 ± 20.88	17.82 ± 3.16	$a^{\#}$, $b^{\$}$
	0	1	500.19 ± 25.53		
	1	2	388.27 ± 12.80	− 1.10 ± 0.62	$a^{\$}$, $b^{\#}$
	1	3	387.49 ± 22.53	− 0.80 ± 6.86	$a^{\$}$, $b^{\#}$
6	*Quercetin dihydrate*	*Kaempferol*			
	1	0	273 ± 7.98		
	1	1	310.60 ± 13.35	10.60 ± 3.00	$a^{\#}$, $b^{\$}$
	2	1	286.94 ± 15.28	17.41 ± 3.74	a^{-}, $b^{\$}$
	3	1	299.36 ± 10.70	13.79 ± 4.08	a*, $b^{\$}$
	0	1	421.32 ± 11.52		
	1	2	339.37 ± 26.28	2.39 ± 5.50	$a^{\#}$, $b^{\#}$
	1	3	358.08 ± 11.89	− 3.05 ± 1.34	$a^{\$}$, $b^{\#}$

Table 1 continued

S.No	Combination of phytochemicals in different ratio		EC_{50} (μM) ± STDEV	Difference in DPPH antioxidant activity (%)	*P*-value
	P1	*P2*			
7	*Quercetin dihydrate*	*Resveratrol*			
	1	0	267.38 ± 1.49		
	1	1	402.71 ± 25.80	8.00 ± 2.45	$a^{\$}$, $b^{\$}$
	2	1	350.46 ± 23.56	19.92 ± 3.34	$a^{\#}$, $b^{\$}$
	3	1	325.95 ± 17.76	25.50 ± 2.35	$a^{\#}$, $b^{\$}$
	0	1	607.63 ± 37.24		
	1	2	461.57 ± 40.83	− 5.36 ± 5.14	$a^{\$}$, $b^{\#}$
	1	3	485.81 ± 37.39	− 10.93 ± 4.07	$a^{\$}$, $b^{\#}$
8	*Hydroquinone*	*Kaempferol*			
	1	0	435.18 ± 4.22		
	1	1	375.71 ± 13.92	12.82 ± 2.87	$a^{\#}$, $b^{\#}$
	2	1	394.56 ± 26.79	8.45 ± 5.86	a^{-}, b^{-}
	3	1	407.99 ± 13.12	5.33 ± 2.69	a*, b^{-}
	0	1	426.63 ± 3.59		
	1	2	384.08 ± 14.40	10.87 ± 2.97	$a^{\#}$, $b^{\#}$
	1	3	391.06 ± 4.91	9.25 ± 0.99	$a^{\$}$, $b^{\$}$
9	*Hydroquinone*	*Resveratrol*			
	1	0	381.06 ± 16.21		
	1	1	430.31 ± 25.90	11.24 ± 3.21	a*, $b^{\$}$
	2	1	405.37 ± 51.99	16.49 ± 8.41	a^{-}, $b^{\#}$
	3	1	418.34 ± 36.26	13.74 ± 5.53	a^{-}, $b^{\#}$
	0	1	588.25 ± 20.43		
	1	2	457.01 ± 43.97	5.77 ± 7.05	a*, $b^{\#}$
	1	3	488.84 ± 50.18	− 0.74 ± 7.45	a*, b*
10	*Kaempferol*	*Resveratrol*			
	1	0	439.63 ± 3.36		
	1	1	517.21 ± 32.37	− 0.4 ± 4.10	$a^{\#}$, b^{-}
	2	1	522.21 ± 25.53	− 1.47 ± 5.82	$a^{\#}$, b^{-}
	3	1	511.68 ± 27.45	0.52 ± 7.22	$a^{\#}$, b^{-}
	0	1	591.04 ± 59.66		
	1	2	545.28 ± 40.40	− 5.74 ± 2.08	$a^{\#}$, b^{-}
	1	3	560.33 ± 45.83	− 8.63 ± 2.75	$a^{\#}$, b^{-}

Variables a and b indicate statistical comparison of EC_{50} values of the phytochemical combinations with phytochemical 1 (1:0) and phytochemical 2 (0:1), respectively

The superscripts *, #, and $ are for P-value ≤ 0.05, 0.01, and 0.001. ' − ' represents non-significant values

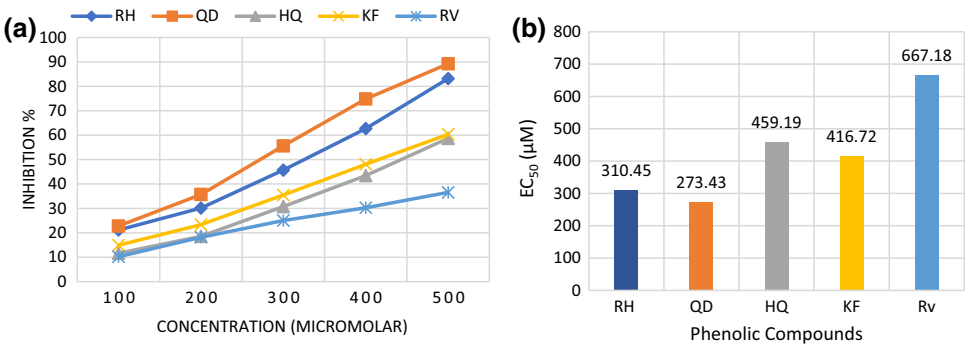

Fig. 2 **a**: DPPH radical scavenging activity (%) **b**: EC_{50} values of selected flavonoid and non-flavonoid phenolic compounds. RH-Rutin hydrate; QD-Quercetin dihydrate; HQ-Hydroquinone; KF-Kaempferol; RV-Resveratrol

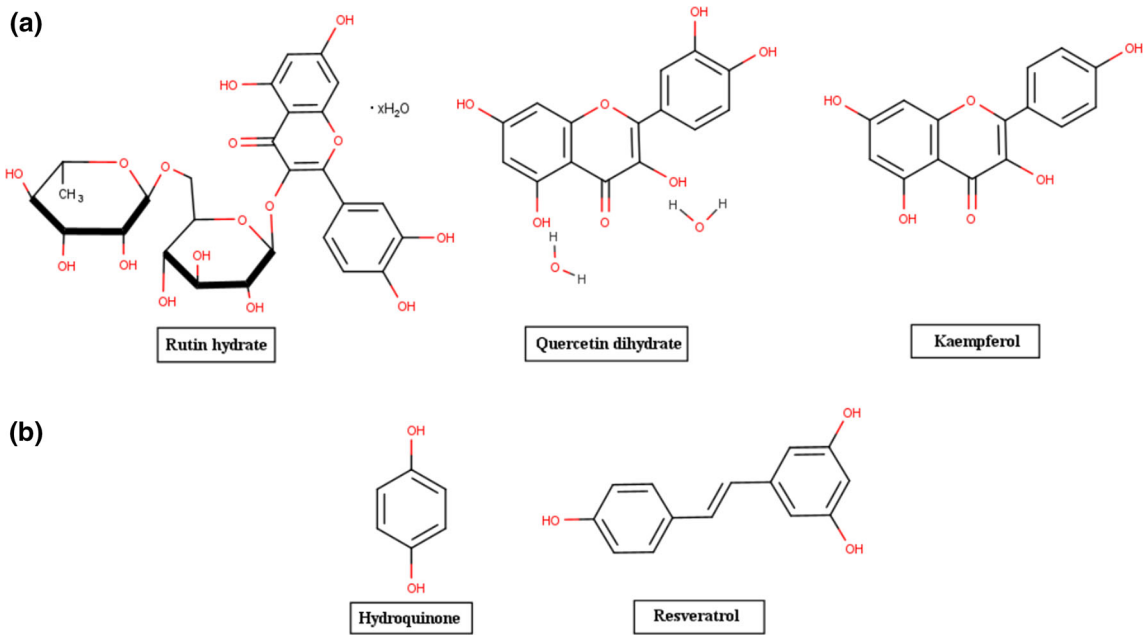

Fig. 3 Chemical structures of **a** Flavonoids, and **b** Non-flavonoid phenolic compounds. Structures are drawn using Marvin sketch software

compounds is generally thought to be due to the hydrogen donating ability of their hydroxyl groups, that is, the more hydroxyl groups are present, the greater the probability of free radical scavenging action. In context of the currently obtained results, it is important to note that quercetin has five hydroxyl groups, whereas all other compounds have less than five hydroxyl groups (Fig. 3), thereby lending credence to the generalized statement above. The availability of hydroxyl groups is influenced by both chemical structure and spatial conformation, which can alter a molecule's reactivity [21]. In the present study, rutin hydrate (glycoside of quercetin) showed less antioxidant activity than its aglycone counterpart (quercetin dihydrate). This was in accordance with other studies [22] that the number of sugar moieties in a flavonoid (in the resulting flavonoid glycosides) as well as their position, all play a significant role in antioxidant activity. Aglycones are often found to have more antioxidant activity than their glycoside counterparts. Even though glycosides are generally considered to be weaker antioxidants than aglycones, a glucose moiety can sometimes improve bioavailability [20].

The individual non-flavonoid phenolic compounds (hydroquinone and resveratrol) had the lowest antioxidant activity. This could be attributed to their different/unique structure and the number of hydroxyl groups (lesser in comparison to quercetin). However, when they were paired with all other flavonoids and with each other, a statistically significant increase in the antioxidant activity was observed for some ratios, thereby producing a synergistic effect (Table 1). This result highlights the importance of considering both qualitative as well as quantitative parameters while designing antioxidant food supplements.

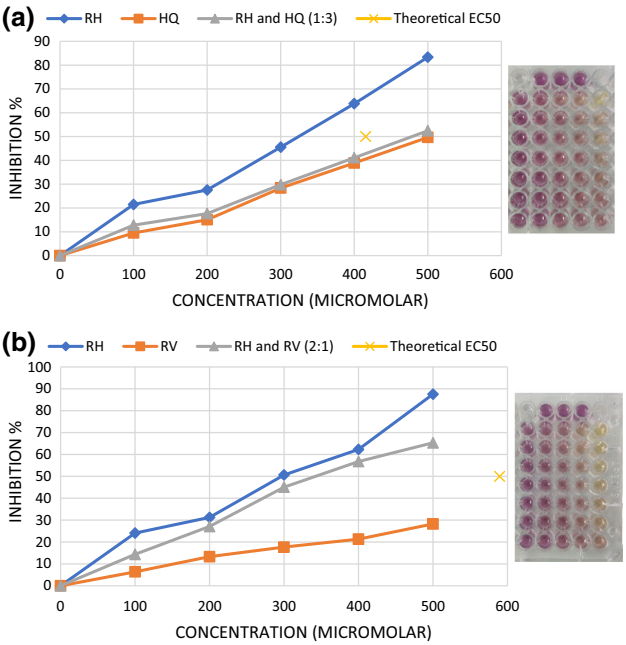

Fig. 4 **a** Antagonistic antioxidant interaction between rutin hydrate and hydroquinone in 1:3 ratio **b** Synergistic antioxidant interaction between Rutin hydrate and Resveratrol in 2:1 ratio. Theoretical EC_{50} is calculated by mixing the individual graphs of both compounds. Theoretical EC_{50} value less than the experimental value shows antagonism, whereas theoretical EC_{50} value more than the experimental value shows synergism. Inset represents a photo of 96 well-plate

Although rutin hydrate and quercetin dihydrate showed the highest individual antioxidant activity among the studied compounds, they did not show any statistically significant synergism. Rutin hydrate showed synergism with kaempferol (3:1, 2:1, 1:1). When the flavonoid kaempferol, with only one hydroxyl group in its B ring, was paired with the non-flavonoid phenolic resveratrol (exhibiting least antioxidant potential in this study), a weak DPPH scavenging activity was observed, indicating statistical antagonism. Rutin hydrate and hydroquinone showed maximum antagonism in 1:3 ratio (Fig. 4a). Maximum synergism was observed when rutin hydrate was paired with resveratrol. However, with an increasing concentration of resveratrol, a significant decrease in synergism was observed (Fig. 4b). It was observed that as the concentration of weaker antioxidants like hydroquinone and resveratrol increases, synergism decreases, that is, the biological activity of the combination of free radical scavenging is decreased.

Conclusion

Some crops, fruits and vegetables such as millets, citrus fruits, grapes, pomegranates, apples, dates, green and yellow vegetables (peppers), cabbage, carrots, dark leafy greens, and banana, have been known worldwide to contain antioxidants [23, 24]. Besides the beneficial antioxidant and medicinal properties of individual phytochemicals, it is being recognized that their combinations, as found in fruits and vegetables, contribute significantly to their nutrition and health benefits [25]. In a representative study, Wang et al. [26] used four different assays and reported on the synergistic antioxidant potential of combinations of—fruit and legume; and, raspberry and adzuki bean. As per the ancient Indian system of medicine known as Ayurveda, certain food combinations are incompatible (*viruddha ahara*) like banana and milk, and are to be avoided for better health [27]. Besides, when phytochemicals are consumed in combinations, their concentration gets balanced thereby offsetting a potential toxic effect of an individual phytochemical consumed in high concentration. For instance, Das et al. [28] showed that the reduced antioxidant activity of decaffeinated green tea could be improved on addition of herbal extract of *Hibiscus sabdariffa*, thereby pointing towards phytochemical synergism.

From the current study, it is evident that the interactions between phytochemicals can be synergistic or antagonistic. Consuming vegetables rich in these compounds in different combinations may contribute to some synergism. In the current study, we have tested combination effects of two phytochemicals at a time which is a simpler glimpse into a more complex phenomenon; but we are aware that models to test effect (as well as bioavailability) of a combination of more than two bioactives (as present in foods) also need to be developed for such studies. Also, customer choices with respect to sensory properties such as taste (besides health benefits) needs to be evaluated for food supplements based on phytochemical combinations [29]. Additionally, before proceeding with cell culture-based studies or animal trials, the pharmaceutical companies need to first test the effect of nutraceutical combinations using in vitro assays (such as DPPH assay used in the current study), as the in vivo studies require greater investment in terms of money and approval from ethics committees.

For production of dietary supplements, the phenolics in foods can be used in particular ratios. Thus, identifying the synergistic combinations of bioactives and optimizing their proportions in the mixture will be a useful effort in the development of functional foods. For instance, juice made from three different sources, such as hemp seeds, pumpkin seeds, and pear juice, showed 52.60% increased scavenging activity and produced a greater synergistic interaction [30]. Still, many phenolic compounds have not been tested for the effect of their interactions on antioxidant activity. Therefore, further research is required for a better understanding of their mechanism of action. Based on such studies, certain food combinations can be promoted (due to synergism) while some can be ruled out (due to antagonism).

Acknowledgements Authors are grateful to the administration of Birla Institute of Technology and Science (BITS), Pilani—Pilani Campus for infrastructural support and encouragement. TJ is grateful to the University Grants Commission (UGC) for providing junior research fellowship.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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