Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 12 [3] February 2023 : 21-25

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Potentiation of Antioxidant Activity of Rutin by Polyamines: *In-Vitro* Studies

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ABSTRACT
Oxidative stress is a disturbed in balance between pro-oxidant and antioxidant levels resulting in to imbalance of generation of reactive oxygen species (ROS), which indirectly affects biological system's detoxification mechanism. Spermine and spermidine are polyamines that have antioxidant properties and are engaged in a number of biological functions. Rutin, a glycoside of the flavonoid quercetin, is found in many plants and fruits and thus accountable for its strong antioxidant activities in both in vitro and in vivo models. Thus considering the therapeutic potential of rutin in many disorders where oxidative stress is an underlying cause and polyamine's potential with the most antioxidant properties due to their enhanced positive charge, it was thought worthwhile to access the in-vitro antioxidant prospective of their combination using various in-vitro antioxidant methods: DPPH radical scavenging, reducing power, and NO reducing activity. In the present study it was found that the antioxidant activity of rutin and polyamines combination was highly significant. As it is shown that, radical scavenging characteristic of polyamines appeared to be co-relatewith numbers of amine groups in the biogenic amines; here spermidine and spermine, which have three and four amino groups respectively, were more effective scavengers which were comparable with the standard used Vitamin C.
Keywords: *Reactive Oxygen Species (ROS), Polyamines, Rutin, Antioxidant*

Received 11.11.2022 Revised 23.01.2023 Accepted 26.01.2023

INTRODUCTION

Oxidative stress is a state of altered redox equilibrium as a consequences imbalance between antioxidants and pro-oxidants. This imbalance causes damage to biomolecules like protein, nucleic acid, DNA and RNA [1]. These disturbances to normal redox state of cells may leads to generation of toxic effects via free radicals production, that ultimately react with cellular constituents and initiates severe damage to the cells. This oxidative stress may impact the normal functioning of the immune system [2], decrease nutrient fascination and metabolism, and weakens the growth performance [3]. It can also cause a number of health disorders, like inflammatory disorders, cancer [4], cardiovascular disorders, diabetes [5], neurological [6] and many other disorders. It has been shown that antioxidants are acting as free radical scavengers, reducing agents, and quenchers of singlet oxygen molecule, and antioxidative enzyme activators in order to ameliorate the impairment caused by free radicals in biological system [7].Compounds that exhibit antioxidant properties like vitamin C, vitamin E, and carbohydrates could be used to preserve food, as cosmetics and pharmaceutical products, as alternative medicine, and for natural medicine and therapies. Various aromatic and medicinal plants and several phytoconstituents have been reported as an important source of natural antioxidants. Chemically, rutin, 2-(3,4-dihyd-roxyphenyl)-5,7-dihydroxy-3-[-L-rhamnopyranosyl-(16)-β,D-glucopyranosyloxy]-4H-chromen-4-1, is a flavonol, richly found in plants, such as passion flower, buckwheat, tea, and apple. Rutin has demonstrated many biological activities like anti-inflammatory, antioxidant, antihypertensive, antiapoptotic, antiautophagic and neuroprotective activities[8].Moreover, rutin has shown protective effects against oxidative stress and lipid peroxidation in in-vivo as well as *in-vitro* conditions[9].Further, it is known to attenuate oxidative stress, enhanced in-vitro production of nitric oxide (NO) and pro-inflammatory cytokines and inhibition of amyloid β (Aβ) aggregation and cytotoxicity [10]. Endogenous polyamine (spermine, spermidine and putrescine) are found in all living organisms and participates in various biological activities, including cell proliferation and differentiation and is alsohaving potent antioxidant

characteristics. Naturally occurring dietary polyamines play a crucial role in human wellbeing, particularly immune system differentiation and development. Polyamine's antioxidant and anti-inflammatory properties may support to prevent chronic diseases like cardiovascular disease, important for cell growth and proliferation, DNA stability, RNA transcription, protein synthesis, immune response modulation, apoptosis, ion channel regulation, especially potassium channel blockage, and antioxidant activity[10]. Polyamine (spermine) is having increased positive charge, known to possess with robust antioxidant effects. Metal chelation, which limits the hydroperoxides creation, leads to deferral of the secondary oxidation product generation, is considered to be a major mechanism of polyamine's antioxidant activity [12, 13]. Among the various polyamines available, spermine acts as free radicals scavenger in nucleous, mitochondria and brain [14-16]. Whereas, spermine and spermidine has been shown to radical scavenging activity on lipoperoxidation *in-vivo* [17]. Therefore, owing to above mentioned reports of polyamines as antioxidants, it was thought worthwhile to have study of polyamines, specially spermine and spermidine in combination with rutin by employing some *in-vitro* antioxidant assays.

MATERIALS AND METHODS

MATERIALS

All the chemical compounds used were of standard quality and procurred from Sigma Chemical Co. (St. Louis, USA), except Spermine tetrahydrocholride, spermidine (Sisco Research Lab, Mumbai, India), and rutin (Yucca Enterprises, Mumbai, India). The other chemicals/ingredients used in present study were of analytical grade and obtained from local suppliers.

METHODS

DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH), an established free radical, most commonly used for evaluation of the radical scavenging activity of antioxidant mediators. The mechanism of action of this procedure is based on decrement of DPPH in methanol solution with existence of a hydrogen donating antioxidant, due to development of the non-radical form DPPH-H.

The free radical scavenging activity of test drugs: rutin, polyamines (spermine and spermidine) and mixture of polyamines+rutin were determined by DPPH scavenging method[18, 19].Briefly, here 0.1mM DPPH solution was prepared in methanol: 39.4 mg of DPPH in 1000 ml of methanol. To 0.5 mL of this solution, 1.5 mL of test compounds (in DMSO) were mixed with varied concentrations (1, 10, 100, 500 & 1000 µg/mL).The resulted solution was vigorously shaken and kept at room temperature for 45 min in dark room. Further, the absorbance was detected at 517 nm (Shimadzu UV-VIS spectrophotometer). Here, vitamin C was used as standard drug for the comparison. The reduction in absorbance by test drugs indicates free radical scavenging effect and was s determined by the formula,

$$\text{DPPH scavenging effect (\% inhibition)} = \{(A_0-A_1)/A_0) \times 100\}$$

Where, A_0denotes absorbance of the control compound, and $A_1$is of test compound and/or standard drug used. The DPPH scavenging activity was expressed as IC_{50} (inhibition concentration) value, that is the concentration of the sample required to scavenge 50% of free radicals existing in the test solution.

Nitric oxide (NO) radical scavenging Activity

Nitric oxide (NO) is an established free radical, and interacts with oxygen or reactive oxygen species. The free radical scavenging characteristics of NO is due to the availability of unpaired electron and is known to demonstrate similar properties like superoxide free radicals. In the present study, NO radical scavenging activity was determined as per the procedure described by Balakrishnan [20]. Briefly, various concentrations of rutin and spermine and spermidine (as 1, 10, 100, 500, and 1000 µg/ml) were prepared in ethanol. To this 0.5 ml of 10 mM sodium nitroprusside in phosphate buffer, equal volume of freshly prepared Griess reagent and 1 ml of various concentrations of test compounds were added, and then the resultant solution were then incubated at 25°C for 3 hours. Further, 100 µl of this reaction mixture was transferred to a 96-well plate, and the absorbance was determined at 546 nm using a microplate reader (Biotek, Italy). Here, ascorbic acid was used as standard drug. The percentage of NO radical scavenging activity of was calculated by the formula,

$$\text{Nitric oxide scavenging activity} = \frac{\text{Absorbance of control-Absorbance of test compounds}}{\text{Absorbance of control}} \times 100$$

Reducing power method assay

The reducing power of test drugs was determined according to the method of Jayaprakasha G. et al.,[21] with some modifications. The test drugs: Rutin, vitamin-C and polyamines were prepared at differing concentrations. One milliliter of each of rutin, vitamin-C and polyamines was mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 30 mmol/l). Then the mixture was incubated at 50°C for 20 min. Further, 2.5 ml of TCA (0.6 mol/l) was added to the mixture,

and centrifuged for 10 min at 3000 g. To the supernatant (2.5 ml) of this solution, distilled water (2.5 ml) and FeCl3 (0.5 ml, 6 mmol/l) was mixed and the absorbance was detected at 700 nm in a UV-spectrophotometer [21].

RESULTS AND DISCUSSION

DPPH radical scavenging assay

Here, the *in-vitro* antioxidant assay, DPPH radical scavenging assay, performed on rutin and polyamine combination reveals significant antioxidant potential as when compared with the vitamin C as a standard. Result of DPPH scavenging activity in the present study indicates that rutin and the polyamines has demonstrated the potent anti-oxidant activity in the concentration dependent manner but the combination of spermine and spermidine with rutin is more significant as shown in table 1. The dose dependent percentile surge in scavenging activity for all concentrations of test and standard drug was observed. The scavenging activity outcome of polyamines, rutin and standard on the DPPH radical was as per the following order: vitamin C>Rutin and polyamines combination > Rutin > spermine > spermidine respectively (Table 1).

Table 1: Comparative anti-oxidant activity of rutin, polyamines and vitamin C

Test compounds	Concentrations (µg/mL)	Scavenging activity (%)			IC_{50} (µg/ml)		
		DPPH radical scavenging	NO radical scavenging	Reducing power assay	DPPH radical scavenging	NO radical scavenging	Reducing power assay
Rutin	1	1.0	1.0	1.0	29.2 ± 0.7	7.9 ± 0.3	19.2 ± 0.5
	10	32.0	6.0	5.0			
	100	73.0	39.0	36.0			
	500	83.0	71.0	80.0			
	1000	88.0	81	84.0			
Spermine	1	10.0	1.0	1.0	31.6 ± 0.5	8.07 ± 0.4	25.6 ± 0.7
	10	39.0	7.0	5.0			
	100	78.0	41.0	30.0			
	500	90.0	66.0	71.0			
	1000	82.0	77.0	79.0			
Spermidine	1	3.0	2.0	1.0	33.2 ± 0.6	8.45 ±0.45	21.2 ± 0.4
	10	11.0	12.0	14.0			
	100	40.0	69.0	75.0			
	500	78.0	72.0	82.0			
	1000	77.0	73.0	85.0			
Rutin + Polyamines	1	3.0	4.0	2.0	23.2 ± 0.6	7.05± 0.25	16.1 ± 0.5
	10	18.0	21.0	23.0			
	100	42.0	49.0	81.0			
	500	88.0	79.0	87.0			
	1000	92.0	85.0	90.0			
Vitamin C	1	5.0	15.0	3.0	16.1 ± 0.4	6.23± 0.25	13.3 ± 0.3
	10	15.0	75.0	19.0			
	100	74.0	95.0	83.0			
	500	94.0	98.0	89.0			
	1000	97.0	99.0	91.0			

Nitric oxide (NO) radical scavenging Activity

In the present study, it was found that the NO radical scavenging activity of rutin and polyamines (spermine and spermidine) was concentration dependent and was as per the following order: vitamin C > Rutin and polyamines combination > Rutin > spermine > spermidine at the highest concentration and the resulting inhibition were of 99%, 85%, 81%, 90%, and 58.8%, respectively.

It has been shown that NO^- is generated in biological tissues by specific nitric oxide synthases, via a five electron oxidative reaction. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO^-. Under aerobic conditions, NO^- reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent [22].

Reducing power method assay

The reducing power is also an extensively used method for assessment of antioxidant potential of phytoconstituents. It is generally characterized by manifestation of reductants that possess antioxidant action via breaching the free radical chains through donation of a hydrogen atom, whereby the test sample diminishes the Fe^{3+}-ferricyanide complex to the Fe^{2+} ferrous form [23]. Therefore, the reducing power of test compound may be examined by quantifying the development of Perl's Prussian blue at

700 nm. In the present study, the iron reducing capacity of the rutin, polyamines (spermine, spermidine), rutin + polyamines and vitamin-C was assessed via ability of these compounds to donate an electron for the reduction of Fe^{3+}-ferricyanide complex to the ferrous form. The reducing ability was as per the following order vitamin C > Rutin and polyamines combination; > Rutin > spermidine > spermine and the IC_{50} values were found to be 13.3±0.3, 16.1±0.5, 19.2±0.5, 21.2±0.4 and 25.6±0.7 respectively (Table 1). All the samples showed a significant concentration-dependent reducing power capacity. It has shown that, polyamines have ability tocreate conjugation with phenolic compounds, and thus affecting their polarity/hydrophilicity, whichin turn may be responsible for their translocation and stability [24].The antioxidant effect of polyamines may be subject to on the number of amino groups in their structure. Therefore, as per this, spermine possesses a greater antioxidant effect than spermidine, and also that of putrescine. Moreover besides to this, polyamine has demonstrated protection of lipids membrane against the damage made by oxidation, and thus leads to maintenance of cells homeostasis [25]. Spermine, a tiny organic cation polyamine, is necessary for the proliferation of eukaryotic cells. Spermine is generally found in the nucleus at millimolar amounts. Spermine has been shown to be a direct free radical scavenger, forming a range of adducts that protect DNA from oxidative damage. Reactive oxygen species cause oxidative damage to DNA, which cells must protect against in order to survive. As a result, spermine is an important natural intracellular molecule that protects DNA from free radical damage [26].Further, it has been reported that spermidine hasrobust anti-inflammatory properties, and also possesses the capacity to reduce age-related oxidative protein damage and the excess of ROS. Thus, result of present study is in agreement with its antioxidant potential [27]. It has been shown that, though the anti-oxidative properties of polyamines remain low compared to the reference antioxidant vitamin C, their ability to bind at various sites at cells susceptible to oxidation, makes them capable macromolecule and thus offering protection from ROS induced oxidative damage[28].

CONCLUSION

Based on the observations in the present study, it is concluded that rutin and polyamines combination exhibited significant antioxidant activity as seen in DPPH radical scavenging, reducing power, and NO radical scavenging assay; and was comparable to that of standard agent, vitamin C. This potentiating antioxidant activity and reducing power capacity of the rutin and polyamines combination may be attributed to their nature as flavonoids, phenolic compound, and chelating agents that may help them to scavenge the free radicals via donating hydrogen or an electron.

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CITATION OF THIS ARTICLE

Patil, Rajesh R., Aswar, Manoj K., Bhise, Satish B. Potentiation of antioxidant activity of rutin by polyamines: *In-vitro* studies. Bull. Env. Pharmacol. Life Sci., Vol 12[3] Feb 2023: 21-25.