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Original Article

IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF RUTIN AND PIPERINE AND THEIR SYNERGISTIC EFFECT

PRAPURNACHANDRA YADALA, A H M VISWANATHSWAMY

Department of Pharmacology, K. L. E. University's College of Pharmacy, Vidyanagar, Hubli 580031, Karnataka, India
Email: vmhiremath2004@gmail.com

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ABSTRACT

Objective: The objective of the present study was to evaluate the antioxidant and cytotoxic activities of Rutin and Piperine and their synergistic effect.

Methods: The Oxidative activity of Rutin, Piperine, and combination of both was confirmed by the level of Superoxide radical (SOD), Nitric oxide (NO), and Reducing power method (RO), Cytotoxic activity was also measured by using brine shrimp lethality bioassay.

Results: The present study showed that combination of Rutin and Piperine showed enhanced antioxidant activity in SOD and NO compared to individuals with IC_{50} values 27.18±0.59 µg/ml and 22.00±0.15 µg/ml respectively and also produced good reducing power activity. All samples showed a potent cytotoxic effect, combinational sample showed a more potent effect than the standard potassium dichromate with LC_{50} 3.96 µg/ml.

Conclusion: Therefore, the present study demonstrated that the combination of Rutin and Piperine exhibits synergistic antioxidant and cytotoxic effect and the study thus provide more insight into the mechanism of the hepato protective action of the combinational sample for the management of hepatotoxicity.

Keywords: Rutin, Piperine, Antioxidant, Cytotoxic, SOD, NO, RO

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INTRODUCTION

Hepatocellular carcinoma (HCC), also called malignant hepatoma is the most common primary liver cancer, which accounts as the third leading cause of deaths worldwide, after lung and stomach cancer [1]. It is responsible for a worldwide incidence of over one million cases annually [2]. HCCs, a fatal malignancy extends in several regions of Africa and Asia (80%) [3]. The majority of HCC cases attributable to primary infections is induced by hepatitis B and C viruses, aflatoxin exposure, cirrhosis, environmental pollutants, obesity, iron overload, and nitrosamines [4]. The current treatment for HCC is employed by several surgical and non-surgical remedies modalities. Potentially beneficial options as surgical resection, liver transplantation, and local ablation therapies are considered when the tumor is restricted to the liver. But, these therapies not only confine to the extent of the tumor, also by the liver disease and circumstance of the patient [5]. In recent years, a large number of natural compounds have been identified and shown to have potential cancer chemo preventive importance due to their strong antioxidant and cytotoxic activities.

Flavonoids, the polyphenolic compounds act as the major nutritional constituents of plant-based food as habitual and folkloric medicine worldwide [6, 7]. Rutin (3ʹ 4′, 5, 7-tetrahydroxy-flavone-3-rutinoside), a common dietary flavonoid with a wide range of pharmacological activities is present in many plants (as buckwheat seeds, tea), fruits (citrus fruits, apple), vegetables (onion), and red wine [8-10]. Different studies have represented the biological effects of rutin, such as anti-oxidative, anti-inflammatory, antihypertensive, anti-carcinogenic, cytoprotective, anti-platelet, antithrombic, anti-diabetic, anti-adipogenic, neuroprotective, hormone therapy and cardioprotective activities [11-13].

An alkaloid, piperine (*Piper longum* L. and *Piper nigrum* L.), is used widely as a traditional medicine for counteractive an assortment of disorders. It is reported to have as an excellent remedy serving in the treatment of gonorrhea, menstrual pain, improving digestion, reducing inflammation, relieving pain and asthma, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions. Besides these, other reported useful effects comprise as diuretic effects, central nervous system depression, relaxation of muscle tension, and alleviation of anxiety [14-16]. Nutritive substances as beta carotene, glucose, amino acids, curcumin, selenium and pyroxidine improve its bioavailability with the aid of piperine [17].

The aim of the present study was to investigate the antioxidant and cytotoxic activities of Rutin and Piperine and their synergistic effect.

MATERIALS AND METHODS

Chemicals

Rutin and Piperine were purchased from Sigma-Aldrich Chemical Company (Bangalore). The other solvents and reagents used in the study were of analytical grade and were freshly prepared in distilled water.

Antioxidant activity of rutin and piperine

Superoxide radical scavenging activity (SOD)

Superoxide anion, a weak oxidant, produces powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [18]. The superoxide anion radical scavenging activity of the sample was investigated by the method of Robak and Gryglewski [19]. In this estimation, 3.0 ml of Tris–HCl buffer (16 mM, pH 8.0), containing 0.5 ml of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract and 0.5 ml Tris–HCl buffer (16 mM, pH 8.0) was added to generate the superoxide anion radicals. Further the reaction is initiated by adding 0.5 ml phenazine methosulfate (PMS) solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and then the absorbance measured at 560 nm against a blank sample. The superoxide anion radical scavenging capacity was calculated using the following equation:

Superoxide anions are scavenging activity % = $[(A_0-A_1)/A_1] \times 100$, where A_0 is the absorbance of the control (dH_2O), and A_1 is the absorbance of the extract/standard.

Nitric oxide scavenging activity (NO)

The formation of NO in biological tissues takes place by metabolizing arginine to citrulline with the help of nitric oxide synthases [20, 21]. Nitric oxide radical scavenging activity was carried out as by addition of 2 ml of sodium nitroprusside (10 mM) to 0.5 ml of phosphate buffer saline (pH 7.4) with 0.5 ml of the sample at various

concentrations (0.2-0.8 mg/ml). Further the mixture was incubated at 25°C, followed by withdrawal of 0.5 ml of incubated solution which was mixed with 0.5 ml of Griess reagent [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at RT for 5 min with 1 ml of naphthyl ethylenediamine dichloride (0.1% w/v)] and then the absorbance measured at 546 nm [22]. The amount of nitric oxide radical inhibition is calculated using the following equation:

% inhibition of NO radical = $[(A_0-A_1)/A_1] \times 100$, where A_0 is the absorbance before reaction and A_1 is the absorbance after the reaction has taken place with Griess reagent.

Reducing power method (RP)

The method as described by Oyaizu [23, 24] was followed where 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of sample dissolved in distilled water. Further, the resulting mixture was incubated at 50 °C for 20 min, followed by addition of 2.5 ml of trichloroacetic acid (10% w/v) which was centrifuged at 3000 rpm (10 min) for the collection of the upper layer of the solution (2.5 ml). It was mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v) and then the absorbance measured at 700 nm against the blank sample.

Brine shrimp lethality bioassay

The method as described [25] was used in this assay. A 24 h LC_{50} bioassay was performed in a multi-well test plate using Nauplii of the brine shrimp *Artemia salina.* This test was conducted as per the standard operating procedure (25±1 °C and 35% salinity) by using three replicates for each sample and ten napulii per replicate. Artificial sea water (ASW) was prepared as described by Kester D. R *et al.*, [26] with composition of chemicals (Sodium chloride, 23.9 g; Sodium sulfate, 4 g; Potassium chloride, 0.67g; sodium bicarbonate, 0.20 g; Potassium bromide, 0.98 g; Boric acid, 0.026 g and Sodium fluoride, 0.003 g) were weighed and dissolved in one liter of distilled water to make ASW. Brine shrimp eggs were incubated in ASW in a two-compartmental plastic tray by providing direct light and warmth (24 to 26 °C) with the help of 60W lamp. After an incubation of 24 h the hatched shrimps moved from one compartment to another compartment of the tray by attracting to light provided by lamp. The hatched napulli were separated from the shells and remaining cysts using a posteur pipette and transferred to fresh ASW. The protocol used in this assay as follows, 10-15 napulii were counted and transferred to six-well plate containing 4 ml of ASW by using posteur pipette. To the wells containing napulii, aliquots from a stock solution of samples were added to make three concentrations viz.100, 500 and 1000 µg/ml. 10 napulii added in 4 ml of ASW was used as positive control, and Potassium dichromate was used as positive control in the experiment. Adjusted the volume with ASW to 5 ml/well and the well plate was incubated for 24 h at room temperature(25 °C–28 °C) and a number of dead napulii were counted after 4 h and 24 h with the help of a magnifying glass. The % of mortality of brine shrimp was calculated from the number of dead nauplii, and the data was analyzed for probit analysis to determine the LC_{50} values and 95% confidence intervals by using SPSS software.

Statistical analysis

Experimental results are presented as means±SEM, and all measurements and analyzes were carried out in triplicate. SPSS V.18.0 statistical software was used for the statistical and graphical evaluations in this study. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons and the Student's t-test. All p-values<0.05 were considered significant.

RESULTS AND DISCUSSION

Superoxide radical scavenging activity

Superoxide produces dangerous hydroxyl radicals; Superoxide anion plays an important role in the generation of reactive oxygen species and singlet oxygen, which causes oxidative damage in lipids, proteins and DNA [27] it has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Also, superoxide has been observed to initiate directly lipid peroxidation [28]. In addition it has been reported that antioxidant activity of some flavonoids are effective mainly via scavenging of superoxide anion radical [29]. Antioxidants are able to inhibit the blue NBT formation [30]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture.

Table 1: Superoxide radical scavenging activity

Concentration(µg/ml)	% of scavenging of rutin	% of scavenging of piperine	% of scavenging of R+P	% of scavenging of ascorbic acid
10	18.02±0.19	27.70±0.33	27.37±2.09	41.13±0.27
25	28.02±0.08	29.65±0.65	39.14±0.80	53.84±0.56
50	55.53±0.85	60.49±0.73	74.49±0.35	83.25±1.40
100	68.10±0.37	73.34±0.55	81.56±0.37	92.41±0.36
200	89.36±0.23	89.45±0.62	91.47±0.25	93.35±0.68
400	97.06±0.14	97.46±0.63	97.91±0.06	99.20±0.13

All the values are expressed as mean±standard deviation; n=3

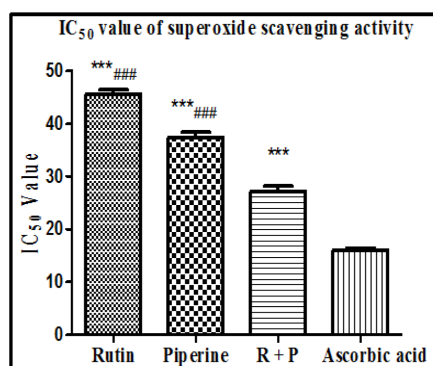

Fig. 1: IC_{50}values of superoxde scavenging activity of rutin, piperine, R+P (Rutin+piperine) and ascorbic acid
Values are expressed as mean±Standard deviation; n=3, by one-way ANOVA followed by Bonferoni multiple comparison tests. Where ******p*<0.001, compared with Ascorbic acid, and ###*p*<0.001, compared with R+P

Nitric oxide scavenging activity

Nitric Oxide is an essential gas required for several normal physiological processes. However overproduction of reactive nitrogen species is a potentially toxic agent with a free radical character it causes nitro sative stress, and this can modify protein structure and affects the normal function of the cells. [22].

In this method, sample solutions were incubated with sodium nitroprusside, which causes nitrite production. The Nitric oxide scavenging capacity of the samples is decreased in the absorption at 540 nm with increases in concentration.

All three samples have exhibited scavenging capacity by decrease in absorbance with increasing in concentration of the sample this showing increase in % of inhibition in nitric oxide scavenging capacity as shown in table: 2

Reducing power activity

For the measurement of reducing ability, the Fe3+ to Fe2+ transformation was investigated in the presence of sample [31].

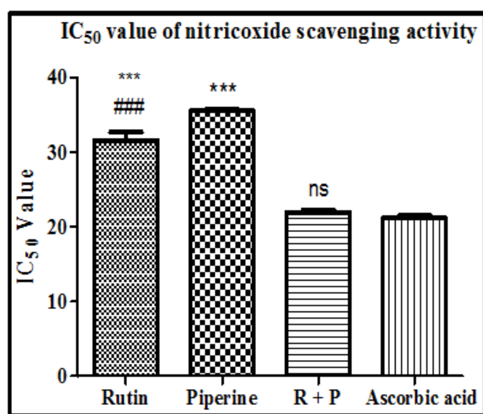

Fig. 2: IC_{50}values of nitricoxide scavenging activity of Rutin, Piperine, R+P (Rutin+Piperine) and Ascorbic acid
Values are expressed as mean±Standard deviation; n=3, by one-way ANOVA followed by Bonferoni multiple comparison test. Where ****p*<0.001, compared with Ascorbic acid, ###*p*<0.001, compared with R+P and *ns*: Non Significant compared with Ascorbic acid

In this assay the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant activity of the samples [32]. The reducing capacity of the sample may serve as a significant indicator of its potential antioxidant activity. Increase in the absorbance the reaction mixture indicates the reducing power of the sample with increases concentrations at 700 nm.

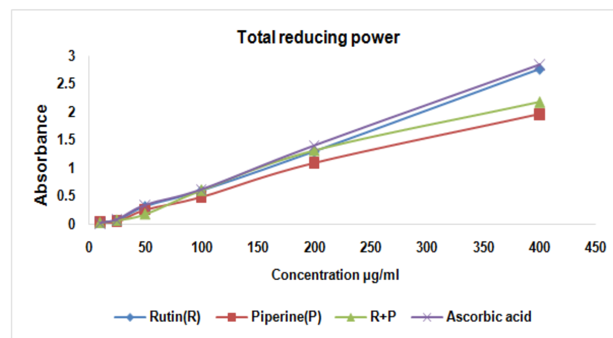

Fig. 3: Reducing power of rutin, piperine, R+P and ascorbic acid

Table 2: Nitric oxide scavenging activity of rutin, piperine, R and P and ascorbic acid

Concentration(µg/ml)	% of scavenging of rutin	% scavenging of piperine	% scavenging of R+P	% of scavenging of Ascorbic acid
10	28.45±1.34	30.86±0.10	37.28±0.29	36.82±0.40
25	46.27±0.04	38.12±0.27	50.64±0.21	52.05±0.20
50	54.11±0.38	57.66±0.12	63.84±0.40	65.47±0.12
100	74.64±0.08	68.72±0.10	78.39±0.16	79.65±0.32
200	84.83±0.73	79.49±0.16	85.74±0.21	86.73±0.26
400	95.56±1.73	92.78±0.20	98.34±0.37	99.45±0.12

All the values are expressed as mean±Standard deviation; n=3

Table 3: Reducing power of rutin, piperine, R+P and ascorbic acid

Concentrations (µg/ml)	Rutin (R)	Piperine (P)	R+P	Ascorbic acid
10	0.035±0.001	0.025±0.005	0.034±0.002	0.020±0.002
25	0.062±0.002	0.045±0.005	0.076±0.004	0.085±0.005
50	0.314±0.004	0.257±0.003	0.186±0.003	0.341±0.005
100	0.595±0.005	0.486±0.003	0.611±0.006	0.620±0.005
200	1.291±0.005	1.089±0.011	1.317±0.003	1.401±0.009
400	2.760±0.013	1.958±0.039	2.177±0.018	2.843±0.024

All the values are expressed as mean±standard deviation; n=3, In this method test compound of R, P and RP have shown good reducing power compared with standard Ascorbic acid.

As shown in fig. in the graph Rutin has exhibited good reducing power activity than Combination of the Rutin and Piperine, however, the combinational compound has shown mild synergistic effect than Piperine alone.

Cytotoxic assay

All samples were screened for cytotoxicity using brine shrimp bench top bioassay. The assay was performed based on the ability of the samples to kill laboratory cultured *Artemia salina* napulii brine shrimp. The assay was done according to protocol reported by meyer *et al.*, [33] and Lincoln *et al.* [34]. Results of the assay are shown in table 4. All samples showed potent brine shrimp larvicidal activity, according to the classification described by meyer *et al.*, crude extracts and pure substances are toxic at LC_{50} value<1000 µg/ml and Non-toxic at LC_{50} value>1000 µg/ml.

All samples were screened at different concentrations viz.10, 100 and 1000 µg/ml and observed for their toxic effect on *A. salina* after 24h incubation. A potassium dichromate was used as a reference standard as described by Padmaja R. *et al.*

Table 4: Cytotoxic activity of rutin, piperine, R+P and potassium dichromate

Tested samples	Concentrations tested (µg/ml)	LC_{50} (24h)#
Rutin (P)	10, 100, 1000	168.04
Piperine (P)	10, 100, 1000	8.06
R+P	10, 100, 1000	3.96
Potassium dichromate*	10, 100, 1000	11.49

All determinations were done in triplicate, 95% confidence limits in probid analysis

Results observed after 24 h of exposure, all samples showed promising toxic effect, Rutin showed 96.66% mortality at 1000µg/ml and Piperine and Combination of Rutin+Piperine has exhibited 100% mortality at 100 µg/ml concentrations, LC_{50} value of Rutin, Piperine and Rutin+Piperine is 168.04, 8.06 and 3.95 µg/ml respectively, whereas potassium dichromate showed 50% mortality at 11.49 µg/ml as shown in table 4.

DISCUSSION

Oxidative stress, a major cause of hepatotoxicity is caused by excessive formation of reactive oxygen species which are by-products of multiple reactions taking in our body.[35,36]. the reactive fee radicals overwhelm the protective enzymes causing destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration [37]. The oxidative imbalance and decrease in endogenous antioxidants leads to the release of elevation of liver enzymes [38,39]. It was considered to evaluate the effect of antioxidant activity.

The strong antioxidant capacity of rutin has been proven by numerous studies; the scavenging activity was widely measured by 2, 2-diphynyl-1-picylhydrazyl (DPPH), N,N-dimethyl-p-phenylen-diamine (DMPD), superoxide and hydrogen peroxide produced from linoleic acid in β-carotene; bleaching assay[12]. Piperine has been reported to exhibit CNS depression, antipyretic and anti-inflammatory activity [40]. piperine has also proved a good bioenhancer with curcumin by enhancing the bioavailability[41]. The additive and synergistic effect of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities [42].

This paper focuses the synergistic effect of rutin with piperine in *in vitro* antioxidant activity. These herbal constituents have an ability to inhibit reactive oxygen species production, thus may enhance its antioxidant and cytotoxic effects. Combination consists of rutin and piperine showed a potent synergistic antioxidant effect on superoxide radical scavenging activity (SOD) and nitric oxide scavenging activity (NO) and the IC_{50} values showed significant ($p<0.001$)in scavenging activity compared to individual results,and rutin exhibits potent ferric-reducing antioxidant power compared to combination of rutin and piperine.

The cytotoxic activity gives the information about anticancer and antitumor potentials of the phytochemicals; the cytotoxic effect was determined by brine shrimps lethality bioassay. The order of cytotoxicity was 1000 µg/ml>100 µg/ml 10 µg/ml. the results showed that Rutin and Piperine showed potent larvicidal activity and results showed similarities with the investigations of Padmaja. R. *et al.*, and Chitali H. Ved., *et al.*, with IC_{50} values 168.04 µg/ml and 8.06 µg/ml respectively. However, the combination of Rutin and Piperine was showed a potent synergistic effect on mortality of the shrimps than individual values and reference standard potassium dichromate with IC_{50} 3.96 µg/ml.

CONCLUSION

The present study results indicate the combination consisting of rutin and piperineis more effective in superoxide scavenging and nitric oxide scavenging activity and rutin showed potent ferric reducing power and also combination of rutin and piperine showed a potent cytotoxic effect on brine shrimp leathality bioassay, enhanced activity could be because of their contribution to decrease the reactive oxygen species formation thus confirming the set of hypothesis.

The study thus provides more insight into the mechanism of the hepatoprotective action of the combinational sample and also provides a scientific basis for its usage in the traditional system of medicine for the management of hepatotoxicity.

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CONFLICTS OF INTERESTS

Declared none

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