

In vitro Antioxidant and Anticancer Activity of Ethanolic Extract of Defatted Rice Bran on MCF-7 (Breast) and A549 (Lung) Cancer Cell Lines

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ABSTRACT

Rice bran, a by-product of milling industries is a nutrient dense material basically used as animal feed. Phytochemicals present in rice bran have potent bioactive compounds that can be used to prevent various non-communicable diseases such as cancer. The widespread prevalence of cancer impels research to look for substances that are anti-cancerous in nature. This study involved the *in-vitro* assessment of antioxidant and anticancer activity of ethanolic extract of defatted rice bran (DFRB). Rice bran was stabilized, defatted and an ethanolic extract was prepared. Phytochemical analysis, total polyphenol content (TPC) and total flavonoid content (TFC) of DFRB was done. Antioxidant activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and hydrogen peroxide scavenging assay. Cytotoxicity was assessed using MTT assay on breast (MCF-7) and lung (A549) cancer cell lines. Phytochemical screening showed the presence of terpenoids, cardiac glycoside, flavonoids, alkaloids, quinone and phenols. The TPC and TFC was 1.15 ± 0.34 mg GAE/g and 4.69 ± 0.40 mg RE/g respectively. DFRB extract showed significant antioxidant activity that was dose dependant, with an IC_{50} value of 7.82 and 111.68 $\mu\text{g/mL}$ for DPPH and hydrogen peroxide scavenging assays respectively. The viability of MCF-7 and A549 cells decreased with the addition of DFRB extract with IC_{50} values of 174 and 145 $\mu\text{g/mL}$ respectively. This study reveals the potential of DFRB extract as an antioxidant and anticancer agent, however a thorough investigation of the underlying mechanisms is required for DFRB to be considered as a therapeutic agent by pharmaceutical industries.

Key words: Defatted Rice Bran, Ethanolic Extract, Anticancer, Antioxidant, Stabilized.

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INTRODUCTION

Rise in non-communicable diseases have emerged as a substantial public health problem in India and other developing countries. Oxidative stress induced diseases have been identified as one of the major causative factors for chronic diseases such as diabetes mellitus, neurodegenerative diseases, cardio vascular diseases and cancer.^[1] The biggest threat to human kind has been identified as Cancer, among several diseases. About

1.8 million cancer cases have been projected in 2025. In India cancer is responsible for maximum mortality with 0.3 million deaths per year. All kinds of cancer have been observed in the Indian population starting from mouth, oesophagus, lungs, breast, stomach, rectum, liver, prostate, cervix, blood etc. In 2018, lung and breast cancers amounted to about 12.3% of the new cases diagnosed. According to data retrieved from WHO mortality database, mortality due to lung and breast cancer is projected to be high in high income countries by 2030, and lung cancer may surpass breast cancer and prevention should be the primary key factor to decrease the incidence.^[2]

Currently, plant-derived antioxidants and their health benefits have been greatly researched. The naturally occurring phytochemicals composed of phenolic

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or polyphenolic compounds have shown to possess antioxidant potential.^[3] Oxidative stress is caused due to the imbalance between the endogenous antioxidant system and free radicals (e.g., reactive oxygen species). Studies have shown that regular intake of dietary antioxidants acts as a protective defence mechanism to the body by removing free radicals and simultaneously guarding the body against oxidative damage.^[4]

In the recent times, much attention has been directed towards the search of bioactive compounds from agro-industrial residues and inclusion in food and pharmaceutical industries as they have shown potential in the prevention of oxidative stress related diseases.^[5] One such abundantly available industrial residue is rice bran, that has been reported to contain several bioactive compounds.^[5] Rice remains the staple food across many Asian countries and while processing paddy, rice bran accounts for about 8%. Rice bran is generally discarded or used as cattle feed after the extraction of oil. However, this defatted rice bran is found to be a good source of phytochemicals which have antioxidant and anticancer properties.^[5]

Research on inclusion of rice bran in diet has shown to have anticancer effects on various types of cancers – breast, lung, liver, and colorectal cancer.^[6] These may be attributed to phytochemicals present in the bran. The bioactive components in rice bran have shown to have a protective mechanism by engulfing the free radicals thereby inhibiting chronic inflammation.^[6] Anticancer activity of rice bran in colorectal cancer has been produced by affecting the colonic tumour environment that modulates the gut microflora and regulating the carcinogen-metabolizing enzymes.

This study aimed at evaluating the *in vitro* antioxidant and anticancer activities of heat stabilized white rice bran (*Sona moosir*)

MATERIALS AND METHODS

Materials

The standards gallic acid, rutin, DPPH were purchased from Sigma Aldrich (St. Louis, USA). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany) and all other chemicals used were of analytical grade. Rice bran was procured from a local mill in Chennai, Tamil Nadu, India).

Preparation of defatted Rice Bran

The impurities were removed using 40 micron sieve and was stabilised by microwave treatment to in order reduce

rancidity by lipase enzymes present in the bran. Rice bran was then subjected to degreasing/ de-fattening using hexane as described by^[7] to produce defatted rice bran. Bran and hexane were taken in the ratio 1:3 (w/v) in a closed container and placed on a magnetic stirrer at 50°C for 30 min. The hexane with fat was discarded and replaced with new solvent and the above step with the same conditions was repeated. After the removal of hexane, the defatted bran was placed in a hood for 18 hr to 24hrs. The obtained defatted rice bran (DFRB) was sieved in a 40 µ mesh sieve and stored in a refrigerator at 4°C until further analysis.

Ethanolic extraction of DFRB

The DFRB sample was subjected to Soxhlet extraction (Borosil) using ethanol as a solvent. The samples (30 g) were packed in a thimble (handmade filter paper). The plant sample filled thimble was carefully placed inside the extractor chamber and poured with selected solvent ethanol as 1:10 ratio. The round bottom flask (reservoir) was heated to 55-60°C in a heating mantle. At least 20 refluxes were run to improve the quality of extract. The resultant solvent extract was condensed using a rotary evaporator (Buchi, Bangalore, India) under reduced temperature in vacuum condition. The resultant extract was collected in a container and stored under 4°C until further analysis.

The extract solution (concentration - 10mg/mL) was prepared by dissolving 200mg of the extract in 1mL ethanol and then made up to 20mL with distilled water in a standard flask.

Qualitative analysis of phytochemicals

The presence of various phytochemicals like saponin, tannin, terpenoids, steroids, cardiac glycosides, flavonoids, alkaloids, anthraquinone, quinone and phenols in DFRB was assessed.^[8]

Estimation of total phenol content

Determination of total phenol content was by the Folin-ciocalteu method with slight modifications.^[9] About 20, 15, 10, 5, 2.5 µg/ mL of standard gallic acid aliquots was pipetted out in to a series of test tubes. To another test tube 100 mg/mL of test solution was taken. All test tubes were diluted with distilled water. To all the tubes 20% Na₂CO₃ was added followed by the addition of Folin-Ciocalteu reagent and kept undisturbed at room temperature for 30 min. The reagent blank was prepared as above without test sample. The readings were read spectrophotometrically at 700nm. The blue colour indicates the presence of phenol.

Estimation of Flavonoid content

Flavonoid content was quantified using aluminium chloride method^[10] with slight modifications. About 100, 75, 50, 25 and 10 µg/mL of standard rutin aliquots was pipetted out in to a series of test tubes. Test solution was prepared by dissolving the ethanolic extract (1mg/mL) in distilled water. To another test tube 1 mg/mL of test solution was taken. All test tubes were diluted with distilled water (2.5 mL). To all the tubes methanol and potassium acetate was added. This was followed by the addition of aluminium chloride solution and incubated for 30 min at room temperature. The reagent blank was prepared without test sample. All samples were read at 510 nm spectrophotometrically. The formation of yellow colour indicates the presence of flavonoid.

Estimation of antioxidant activity of DFRB by 1,1 – diphenyl – 2- picrylhydrazyl (DPPH) assay

Stock solution of DPPH (1mM) was prepared in methanol. Aliquots of ethanolic extract of the DFRB solution were pipetted out in various concentration in to series of test tubes (50, 100, 150, 200 and 250 µg/mL). All the test tubes were made up to 1.0 mL with distilled water. To all the tubes 2 mL of DPPH was added and kept undisturbed at room temperature in darkness for 10 min. The change in colour from deep violet to colourless was observed and read spectrophotometrically at 520 nm.^[9] The reagent blank was prepared without test sample. Ascorbic acid was used as the reference standard.

Estimation of antioxidant activity of DFRB by hydrogen peroxide radical scavenging assay

This assay was performed according to the method of Sundaram *et al.*^[11] with modifications. Hydrogen peroxide (40 mM) solution was prepared in phosphate buffer (pH 7.4). About 100, 200, 400, 600, 800, and 1000 µg/mL of test solution aliquots were pipetted out into a series of test tubes. The volume of all the tubes were made up to 1.0 mL with distilled water. To all the tubes 40mM hydrogen peroxide was added and kept undisturbed at room temperature for 10 min. The decrease in the colour intensity of the sample, reflected the hydrogen peroxide scavenging activity which was read at 230 nm. A reagent blank was prepared without the test sample. Butylated hydroxytoluene was taken as the standard.

In vitro anti-cancer activity of DFRB extracts

DFRB extracts were tested for the cell growth inhibition property on MCF-7 and A549 cell lines using

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay following the method described by Dantu *et al.*^[12] All the cell lines were purchased from National Centre for Cell Science, Pune. The cell lines were pre-grown in a Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin. For the experiment, the cells were grown in 25 cm ×25 cm ×25 cm tissue culture flasks containing DMEM at 37°C under a humidified atmosphere of 95% air and 5% CO₂. The stock solution of DFRB extract was prepared at a concentration of 10 mg/mL. The working stock was prepared in complete DMEM supplemented with 10% FBS and antibiotics. MTT solution (5 mg/mL) was prepared in PBS and after 48 hr incubation, 50 µL of MTT solution was pipetted into each well to achieve 1mg/mL as final concentration. The plate was further incubated for 3 hr in incubator and the medium was carefully decanted by pipetting. The formazan crystals were air dried in dark place for 30 min at room temperature and dissolved in 100 µL Dimethyl sulfoxide. The plates were mildly mixed at room temperature for 10 minutes and the Optical density (OD) was measured using Synergy HT microplate reader at 570 nm. From the optical densities the percentage growths were calculated using the following formula:

$$\text{Percentage growth} = 100 \times [(T - T_0) / (C - T_0)]$$

Where,

T is optical density of test,

C is the optical density of untreated control,

T₀ is the optical density at time zero (at the time of compound addition).

From the percentage growths a dose response curve was generated and GI₅₀ values were interpolated from the growth curves.

Statistical analysis

All analysis was done in triplicates and expressed as mean ± standard deviation. Two-way ANOVA and Pearson's correlation coefficient was calculated using statistical tool Graph pad Prism 5.

RESULTS

Yield of defatted rice bran

The yield of defatted rice bran ethanolic extract is presented in Table 1. The extraction was carried out using a 30g sample with a solvent volume of 300 mL to yield an extract of 28.71%.

Phytochemicals in DFRB

Rice bran contains phytochemicals with promising health benefits.^[13] DFRB extract showed the presence of terpenoids, cardiac glycoside, flavonoids, alkaloids, quinone and phenols (Table 2).

The concentration of total phenols in DFRB extract was 1.15 ± 0.34 mg GAE/g, while the total flavonoids was found to be 4.69 ± 0.40 mg RE/g of DFRB extract.

Antioxidant activity of DFRB

The antioxidant activity by DPPH is depicted graphically in Figure 1. The IC_{50} Value for the given sample for radical scavenging assay was $7.82 \mu\text{g/mL}$. (IC_{50} was calculated using non-linear regression model using graph pad)

Hydrogen peroxide scavenging activity is represented in Figure 2. The IC_{50} value for the DFRB extracts was $111.68 \mu\text{g/mL}$.

Anticancer activity of DFRB

The ethanolic extract of DFRB was tested against MCF-7 and A549 cell lines to evaluate growth inhibition properties. The test compound of each concentration was performed in triplicates and cumulative variation was maintained less than 20% between the data points. The anticancer activity is depicted in Figure 3.

Table 1: Yield of ethanolic extract of Defatted Rice Bran (DFRB).

Sample (g)	Solvent (mL)	Yield (g)	Yield (%)
30	300	8.61 ± 0.61	28.71

Mean \pm Standard deviation of three independent estimations

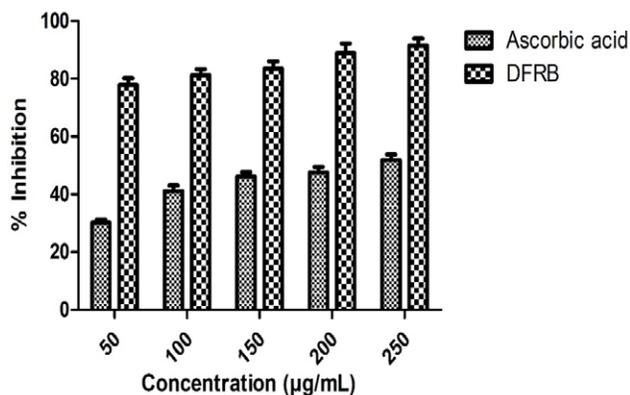


Figure 1: Antioxidant activity of ethanolic extract of DFRB by DPPH assay.

DFRB – defatted rice bran
Mean \pm Standard deviation of three independent estimations

Cytotoxicity is indicated by IC_{50} . This is the amount of concentration that could inhibit 50 percent of cancer cell growth. The results showed that ethanolic extract of DFRB showed IC_{50} values of $174 \mu\text{g/mL}$ for MCF-7 and $145 \mu\text{g/mL}$ for A549.

DISCUSSION

The present study utilised heat stabilised defatted rice bran for the sequential extraction of crude phytochemicals from rice bran with ethanol as a solvent. A 28.71% extract was obtained from 30 g of the DFRB sample which was studied for the analysis of various properties.

Phytochemical screening of the DFRB extract showed the presence of secondary metabolites such as phenols, flavonoids, terpenoids, alkaloids and cardiac glycosides (Table 2). In a study by Moko *et al.*^[14] the non-coloured rice bran (*Supervin* and *Cigeulis*) and red varieties were screened for phytochemicals. All the three varieties showed the presence of phenolic compounds, flavonoids, triterpenoids, alkaloids, and saponins. Rice bran phytochemicals exist as glycosides (linked to sugar moieties) or as complexes coupled to lipids, carbohydrates, phenols, organic acids, and amines.^[15] These phytochemicals (lipophilic in nature) are considered as potential antioxidants that possess various health benefits in the reducing the oxidative damage and prevention of cardiovascular disorders and cancers.^[15]

Polyphenols play an important role as antioxidant, anticarcinogenic and antimutagenic agents.^[3] The total phenolic content of the DFRB was found to be 1.15 ± 0.34 mg GAE/g. An Indonesian variety had 2.66 mg GAE/100 g^[16] and some Iranian varieties *Fajr* and *Tarem* rice bran ethanolic extract possessed $1.67 \pm$

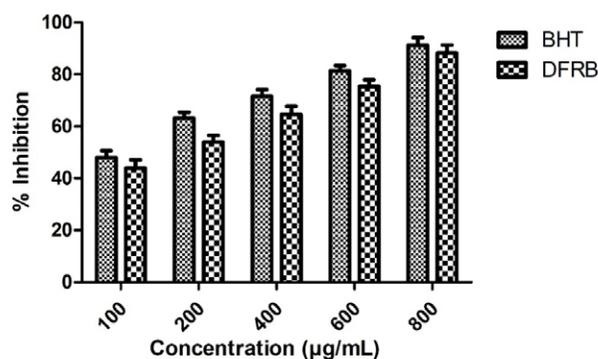


Figure 2: Antioxidant activity of ethanolic extract of DFRB by hydrogen peroxide radical scavenging assay.

Mean \pm Standard deviation of three independent estimations
BHT – Butylated hydroxy toluene; DFRB – defatted rice bran

0.01mg GAE/g of bran and 1.05 ± 0.02 mg GAE/g of bran respectively. The phenol content of rice bran in the present study was lesser than the local varieties used in Iran.^[17] The variations could be attributed to the variety, methodology and the type of solvent used for extraction.

Flavonoids are another group of secondary plant metabolite that play a vital role in plants, animals and bacteria. DFRB extract showed a flavonoid content of 4.69 ± 0.40 mg RE/g. A study by Shen *et al.*^[18] observed that among the TFC of red, white and black rice bran varieties, the least flavonoid content was found to be white rice bran. This statement is in agreement with that of Srisawa *et al.*^[19] showing that pigmented varieties of rice bran have better presence of flavonoids than the non-pigmented white rice bran varieties. The solvent used for extraction affects the yield of the phytochemical constituents. Ghasemzadeh *et al.*^[20] studied the ethanolic (60% v/v) extract of defatted rice bran of red and brown varieties and showed highest total flavonoid content (TFC) values (90 and 70 %), than other solvents like acetone (70 %) and water (30 %). The high yield in ethanol medium could be attributed to the similar polarities of the solvent and the flavonoids present in the rice bran.^[20] Similarly, it was seen that among rice bran, rice husk and ground rice, the husk had the highest TFC content due to its reduced particle size.^[19]

Antioxidant activity is defined as those compounds that are capable of protecting any biological system against the harmful effects caused by the excessive reactive oxygen species (and nitrogen) (RONS). The most commonly used method to measure the antioxidant activity is DPPH. DPPH is the most stable organic radical that is widely used. In this reaction the antioxidants react with it and convert the violet coloured stable radical into

yellow coloured α,α -diphenyl- β -picrylhydrazine.^[21] The change in colour can be taken as an indication for the extracts scavenging activity.

The DPPH activity represented in Figure 1 explains that, on increasing the concentration DFRB extract from 50 to 250 $\mu\text{g/mL}$, the radical scavenging power increased significantly ($p < 0.05$). The least concentration of the extract (50 $\mu\text{g/mL}$) had 70 % inhibition, whereas at 250 $\mu\text{g/mL}$, 91% of inhibition was observed. The antioxidant activity of DFRB extract was significantly higher ($p < 0.05$) than that of the standard ascorbic acid (50%). The high antioxidant activity of rice bran could be attributed to the varied phytochemicals present in it. This is in accordance to the results observed by Reza *et al.*^[22] where various DFRB extracts were used to study the DPPH activity.

The high antioxidant activity of DFRB could be due to heat stabilisation step that reduced oxidation. Mariod *et al.*^[23] observed in their study that heat stabilized rice bran had significantly higher antioxidant properties when compared to unstabilized rice bran samples. A low correlation was seen between the phenolic ($r = 0.32$) and flavonoid ($r = 0.47$) content with the DPPH activity. In biological systems superoxide dismutase acts on superoxide radical and is converted to hydrogen peroxide, which is extremely reactive in the presence of certain metals, and the hydroxyl radicals are very potent ROS that can attack DNA molecules.^[24] The hydrogen peroxide radical scavenging assay of DFRB extract is shown in Figure 2 exhibits an efficient activity in reducing the ROS species. With an increase in concentration of the extract. The inhibition property was

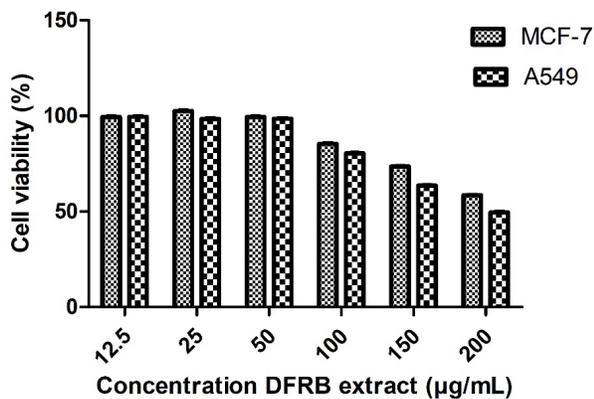


Figure 3: *In vitro* anticancer activity of ethanolic extract of DFRB.

DFRB – defatted rice bran
 Mean \pm Standard deviation of three independent estimations

Table 2: Phytochemical analysis of DFRB extract.

S. No.	Parameters	Results
1.	Saponin	Absence
2.	Tannin	Absence
3.	Terpenoid	Presence
4.	Steroids	Absence
5.	Glycosides	Absence
6.	Cardiac glycosides	Presence
7.	Flavonoids	Presence
8.	Alkaloids	Presence
9.	Anthraquinone	Absence
10.	Quinone	Presence
11.	Phenol	Presence

significantly ($p < 0.05$) enhanced up to 85%. However, the standard BHT exhibited greater inhibition of free radicals (95%) when compared to the sample (85%). A significant positive correlation was seen between the total polyphenol ($r = 0.741$), total flavonoid contents ($r = 0.85$) of the DFRB sample and its hydrogen peroxide scavenging activity.

In a study by Reza *et al.*^[22] ethanolic extract of rice bran showed highest antioxidant activity when compared with other extracts. It was seen that as the concentration of the sample increased, the H_2O_2 scavenging of the sample was in par with the standard. This study shows that DFRB was very much effective in quenching the free radicals.

Similar study by Zaky *et al.*^[25] showed the antioxidative ability of defatted rice bran with different mechanisms - DPPH (56.56%), ABTS (45.34%), and a metal chelating activity (53.14%) respectively. According to Devi *et al.*^[26] the phytochemical constituents of defatted rice bran extract i.e., ferulic acid, triclin and oryzanol could exhibit a synergistic effect for the observed antiradical efficacies.

Exhaustive research is being carried out in search of anticancer compounds from natural food products, one such product being rice bran. The bioactive components of dietary rice bran have been shown to inhibit carcinogenesis by apoptosis, anti - proliferation of cancer cells, scavenging free radicals, suppressing chronic inflammation and blocking cell-signaling pathways.^[6]

The anticancer effect of ethanolic extract of defatted rice bran on the breast cancer cell lines depicted in Figure 3 clearly shows that with increasing concentration of the DFRB extract the cell viability decreased from 100% to 50%. A549 lung cancer cell line exhibited a higher antiproliferative effect than MCF-7. Similar results were observed by^[27,28] which provides significant evidence on the intrinsic safety of dietary rice bran in the treatment of cancer. Dietary rice bran as a whole extract encompasses a complex mixture of phytochemicals having a unique potential to interact with several cellular targets that helps in the prevention cancer.^[6]

Studies have shown that methanolic extract of white rice bran cultivar *Hommali 105*, inhibited the growth of cancer cells in the following order – prostate, cervical and breast cell lines.^[29] A study by Rukmana *et al.*^[30] showed that, among 4 Thai rice varieties (3 black rice bran and 1 white rice – IR 64) ethanolic extract of IR 64 showed cytotoxic effects in Vero cell lines, whereas in the present study, there was no cytotoxicity observed with the ethanolic extract of DFRB. Rice by-products had shown significant activity against breast tumour cell

lines. The effects were attributed to the synergistic effect of ethanolic extracts and phenolic compounds present in these by-products.^[31]

CONCLUSION

The study was carried out to analyse the antioxidant and anticancer properties of ethanolic extract of defatted rice bran (DFRB). The DFRB extract showed the presence of various phytochemicals such as flavonoids and phenols with the flavonoid content being slightly more than the phenol content. The antioxidant potential was evident from DPPH and hydrogen peroxide scavenging assays. The anticancer activity of ethanolic extract of DFRB was significant on breast and lung cancer cell lines. The results conclude that defatted rice bran can be undertaken for further research to analyse its efficacy against cancer treatment. Bioactive compounds have received a significant attention from consumers; defatted rice bran is showing promising therapeutic effects and thus it can be taken into consideration by both food and pharmaceutical industry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

DFRB: defatted rice bran; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **BHT:** butylated hydroxy toluene; **TPC:** total polyphenol content; **TFC:** total flavonoid content; **GAE:** gallic acid equivalents; **RE:** rutin equivalents.

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