Antioxidants and Cytotoxicity Analysis of Coconut Husk Extract

Heenataj. B, Kushmitha. V, Dr. N.G. Ramesh Babu, Dr. I. Seethalakshmi

***Abstract*— *Cocos nucifera* L. (family Arecaceae) commonly known as coconut which is considered as an important fruit crop in tropical countries. It is well known for its effective properties such as antitumor, antioxidant, antimicrobial, antidotal, antiseptic, bactericidal, etc. Our objective was to determine the antioxidant property and cytotoxicity of the husk extract. The analysis was carried out for antioxidant activity by DPPH (1,1 – Diphenyl -2- picrylhydrazyl) assay and FRAP(Ferric reducing antioxidant power) assay along with super oxide dismutase (SOD) and catalase assays. Later, ethanol-coconut husk extracts were evaluated for cytotoxicity by using MTT assay on *Vero* cells. This study demonstrates that the husk extract has antioxidant effect and cytotoxic property.**

Index Terms—**1,1 – Diphenyl -2- picrylhydrazyl(DPPH), Dimethylsulfoxide(DMSO), Butylated hydroxytoluene (BHT), Ferric reducing antioxidant power (FRAP), 2, 4, 6- Tris (2-pyridyl)-s-triazine (TPTZ), Superoxide dismutase (SOD), Foetal bovine serum (FBS).**

I. INTRODUCTION

Cocos nucifera L. (family Arecaceae) is commonly known as coconut, considered as an important fruit crop in tropical countries. Coconuts are unique in terms of their fruit (a drupe) morphology. The most interesting feature of the fruit is its wall. The fruit wall comprises of three layers exocarp, mesocarp, and endocarp.

Due to extensive cross linking between phenolics, lignin and polysaccharides, the mesocarp becomes hard and fibrous. Fibrous coconut fruit is not only edible but also suitable for multipurposes. As a traditional medicine in northeastern Brazil, coconut husks have been used for the treatment of diarrhoea and arthritis. Heating the coconut shells gives an oil that is used against ringworm infections in India. The use of natural products with therapeutic properties is as ancient as human civilization. Even though the pharmaceutical industries have produced many new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased.

Some plants are used popularly for malaria treatment in Nigeria has been scientifically authenticated. In addition, the scanty oil which oozes out while burning coconut shell is used to treat malaria in Nigeria (Adebayo et al., 2012). In addition, the alcoholic extract of ripe dried coconut shell possesses antifungal activity attributed to the high content of phenolic compounds. The aqueous extracts from the husk fibers of *Cocos nucifera* present antibacterial, antiviral, antileishmanial, antinociceptive and free radicals scavenging activities; *Cocos nucifera* extracts have antiproliferative effects on lymphocytes and the brown, dry and very fibrous husk presents a high content of pentosans, cellulose and lignin, whereas its aqueous extract is composed of catechin and epicatechin together with condensed tannis (B-type procyanidins).

Since catechins are powerful inhibitors of cellular growth, presenting anticancer, antimutagenic, antibacterial, antiviral and anti-inflammatory activities, they may be the main phytochemicals responsible for the biological activities of *Cocos nucifera* husk fiber.

The alcoholic extract of ripe dried coconut shell has antifungal activity against *Microsporum canis, M. gypseum, M. audouinii, Trichophytonmentagrophytes, T. rubrum, T. tonsurans*and *T. violaceum.* The activity was mainly attributed to the high content of phenolic compounds (sunglasses *et.al*., 2011). However, studies regarding the polyphenol content of the coconut fruit wall are limited.

Plant phenols are of interest because they are an important group of natural antioxidants and some of them are potent antimicrobial compounds.

An antioxidant is an agent that inhibits oxidation; any of numerous chemical substances including certain natural body products and nutrients that can neutralize the oxidant effect of free radicals and other substances. Free radicals are normally scavenged from tissues by the antioxidant enzymes superoxide dismutase and glutathione peroxidase.

In the recent past, research on antioxidants has increased considerably (Kusano et al., 2003). Due to the involvement of different complex mechanisms, accurate and quantitative measurement of antioxidant properties by a simple and universal method is not possible. The mechanism of antioxidant activity in vitro may involve direct inhibition of reactive oxygen species or the scavenging of free radicals (Dini & Tenore 2006). Thus, DPPH, FRAP assays have been used to evaluate the antioxidant property of the methanolic extract from the mesocarp. In continuation to this study, it has been reported about antioxidants and cytotoxic activity of coconut husk extract.

Cytotoxicity is the ability of being toxic to cells. Testing the effects of compounds on the viability of cells grown in culture is widely used as a predictor of potential toxic effects in whole animals. For any newly discovered drug it becomes necessary to study about its cytotoxic property to know about its level of safety to humans. The present study aimed to assess the cytotoxic activity of coconut husk extract was carried out by

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using Ethanol extracts that were extracted for cytotoxicity by using MTT assay.

II. THEORY

A. *Materials and methods*

SOXHLET PROCEDURE

The sample was washed with distilled water to remove any adherent particles, shade dried and powdered. 25g of sample was weighed and extracted with 300ml of ethanol by continuous hot percolation with the help of Soxhlet apparatus for 10hrs of time. On completion, the extract was filtered and concentrated using rotary evaporator under reduced pressure and controlled temperature of 50^0C – 60^0 C. The concentrates were stored in the refrigerator for further use.

III. ANTIOXIDANT ASSAY

DPPH ASSAY: (Molyneux, 2004)

DPPH (1,1-diphenyl-2-picrylhydrazyl) is characterized as a stable free radical by the delocalization of the spare electron over the molecule, so that the molecules do not dimerize, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centered at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Blois,1958) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present).

Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Z• + AH = ZH + A•

where ZH is the reduced form and A• is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant.

CHEMICALS

1,1 – diphenyl -2- picrylhydrazyl (DPPH)
Dimethylsulphoxide (DMSO)
BHT (standard)-1.6mg/ml in methanol

Samples desired concentration from 1 mg /ml –max of 5mg / ml (in /DMSO)

DPPH INTRODUCTION

Aliquot 3.7 ml of absolute methanol in all test tubes and 3.8ml of absolute methanol was added to blank.

Add 100µl of BHT to tube marked as standard and 100µl of respective samples to all other tubes marked as tests. 200µl of DPPH reagent was added to all the test tubes including blank. Incubate all test tubes at room temperature in dark condition for 30 minutes. The absorbance of all samples was read at 517nm.

Table 1: Procedure for DPPH Assay

S.NO	REAGENTS	BLANK	STANDARD	TEST
1	Methanol	3.8ml	3.7ml	3.7ml
2	BHT	-	100µl	-
3	Sample	-	-	100µl
4	DPPH	200µl	200µl	200µl
Incubation at dark for 30 minutes				
Optical Density at 517 nm				

CALCULATION

$$\% \text{ Antioxidant activity} = \left(\frac{\text{(Absorbance at blank) - (Absorbance at test)}}{\text{(Absorbance at blank)}}\right) \times 100$$

FRAP ASSAY

Total antioxidant activity is measured by ferric reducing antioxidant power assay of Benzie and Strain (1999). The FRAP assay, is presented as a novel method for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.

Procedure for FRAP Assay

100 µl of sample was added to the tube marked test and 3 ml of FRAP reagent was added to it.

3 ml of FRAP reagent was taken as a blank.

Absorbance is measured at 0 minutes after vortexing at 593 nm.
Samples are then placed at 37ºC in water bath and absorption is again measured after 4 minutes.
Ascorbic acid was used as the standard.

Table 2

S.No.	Contents	FRAP ASSAY	
		Blank	Test
1.	Sample	-	100µl
2.	Working FRAP Solution	3ml	3ml

CALCULATION
FRAP value of sample (µM) = (Change in absorbance of sample from 0 to 4 minute / change in absorbance of standard from 0 to 4 minutes) x FRAP value of standard (1000 µM)
Note: FRAP value of ascorbic acid is 2.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)
Superoxide dismutase in the liver tissue was assayed by the method of Kakkar *et al.* (1984). The assay is based on the inhibition of the formation of NADH-phenazinemethosulphate-nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 secs, addition of glacial acetic acid stops the reaction. The color developed at the end of the reaction was extracted into n-butanol layer and measured in a spectrophotometer at 520 nm.

PROCEDURE
0.5 mL of tissue homogenate was diluted to 1.0 mL with water followed by addition of 2.5 mL of ethanol and 1.5 mL of chloroform (chilled reagents were added). This mixture was shaken for 90 secs at 4 °C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazine methosulphate and 0.3 mL of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30 °C for 90 secs, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL n-butanol. The mixture could stand for 10 min; centrifuged and n-butanol layer was separated. The color density of the chromogen in n-butanol was measured at 510 nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.
SOD activity was expressed as Unit/min/mg of protein

ESTIMATION OF CATALASE (CAT)
The activity of catalase was determined by the method of Sinha (1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. The catalase preparation could split H_2O_2 for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H_2O_2 as chromic acetate was determined .

Procedure
To 0.9 mL of phosphate buffer, 0.1 mL of supernatant and 0.4 mL of hydrogen peroxide was added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 mL of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was read at 620 nm.
Catalase activity was expressed as Γmol of H_2O_2 consumed/ min/mg of protein.

IV. CYTOTOXICITY ACTIVITY

Cell line and culture
Cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

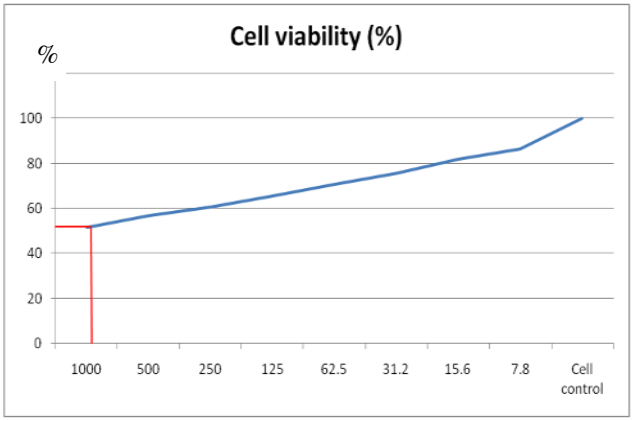
in Vitro assay for cytotoxicity activity:(MTT assay) (Mosmann, 1983)

Cells (1 × 10^5/well) were plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

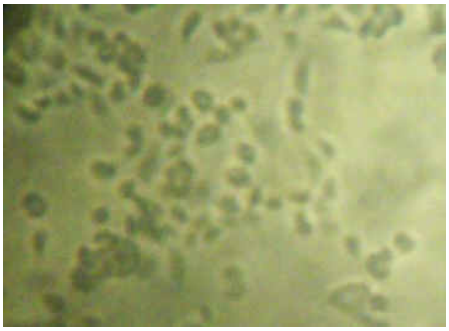
Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments

V. RESULTS



Concentration (µg/ml)

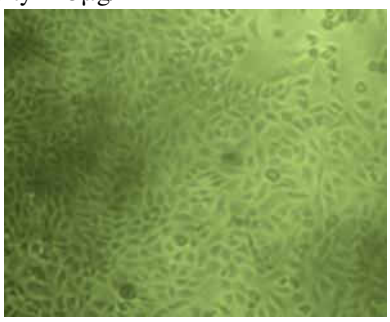
Normal Vero cell line
Toxicity-1000µg/ml



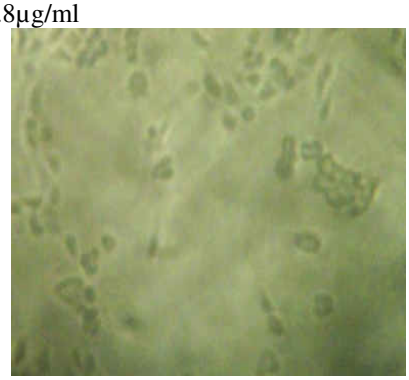
Results and discussion

S.no	Sample	Value
1.	DPPH	41.14%
2.	FRAP	390microMolar
3.	SOD	57.74%
4.	Catalase	7.15 moles
5.	MTT	51.62%

Cytotoxicity effect of Coconut husk on *Vero cell* line
Toxicity-125µg/ml



Toxicity-7.8µg/ml



CONCLUSION

We conclude our study by saying that *cocus nucifera* extract has the antioxidant and cytotoxicity property which is helpful for therapeutic purposes.

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