

FREE RADICAL QUENCHING EFFICACY OF VARIOUS EXTRACTS OF *COSTUS PICTUS* TO COMBAT OXIDATIVE DAMAGE/STRESS: AN *IN VITRO* STUDY

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ABSTRACT

Objectives: The objectives of this research article are to elucidate the antioxidant activity of the sequentially extracted fractions using solvents with a varying range of polarity from the leaves of *Costus pictus* (*Zingiberaceae*). The antioxidant efficacy to combat the oxidative stress was evaluated based on their superoxide, hydrogen peroxide, and nitric oxide (NO) scavenging activity of the leaf extract.

Methods: Extraction was performed by sequential extraction methods with low to high polar solvents. Using hexane as a low polar, dichloromethane as medium polar, and ethyl acetate and methanol as high polar, based on their individual boiling points extraction was carried out. The extracts were then evaporated using a rotary evaporator under vacuum and stored in the dry container. Then, antioxidant activity of each extract was evaluated for superoxide, hydrogen peroxide, and NO scavenging activity, ascorbic acid was used as a standard drug for the study of antioxidant activity. The output was statistically interpreted, and the most significant concentration of the best extract with good antioxidant activity was evaluated.

Results: As the methanolic solvent is highly polar and able to retrieve the active lead components from the plant material, was very well correlated with the higher degree of free radical scavenging efficacy. It also exhibited the most and significant inhibition in superoxide radical scavenging, hydrogen peroxide scavenging, and NO scavenging which was about 86 ± 1.23 , 94 ± 0.34 , and 86 ± 1.87 at 250 $\mu\text{g/ml}$ of extract, respectively. The results from our experiments were in par with the positive control chosen.

Conclusions: The above results help us to substantially conclude that bioactive components are extracted well in high polar solvents such as methanol, which mainly includes flavonoids and related polyphenols. These compounds present in *C. pictus* may be an active lead for potent antioxidant activities which would need further investigations in molecular level.

Keywords: *Costus pictus*, Hexane, Dichloromethane, Ethyl acetate, Methanol, Ascorbic acid.

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INTRODUCTION

Oxidative stress encounters the biological system only when the generation of unstable reactive oxygen overcomes the body's ability to carry out detoxification of these reactive species. This imbalance in the production leads to oxidative damage to macromolecules such as proteins, lipids, and DNA within the body [9,10]. Since this continues as a chain reaction, it bothers the neighborhood cells thereby causing severe oxidative stress or damage and starts to spread [3,6]. Free radicals occur naturally within the body within certain limits where they had noble effects within the system such as killing of invading pathogens. However, any excessive production and unwanted ions could be demagnetized by the enzymatic antioxidant system present within the cells [2]. Certain external factors that can trigger the production of these damaging free radicals include exposure to ultraviolet, pollution smoking, and eating habits. Natural antioxidants, such as superoxide dismutase, catalase, and a family of peroxidase enzymes, are the enzymatic pool for fighting against free radicals [7].

These antioxidants carried out the demagnetization process of the free radicals, thereby rendering them neutrally charged to other nearby cells. In any diseased condition, the antioxidants produced naturally by the body are not enough to neutralize all of the free radicals in the body [11,15]. Therefore, a regular dietary supplement is essential to reduce oxidative stress and related damage. Antioxidants have the remarkable ability to detoxify or neutralize damaged molecules by donating hydrogen atoms to the molecules without destabilizing themselves [13]. Some antioxidants even have a chelating effect on free radical production that's catalyzed by heavy metals. In addition, it has

been determined that antioxidant effect of plant products is mainly due to active phytoconstituents such as flavonoids, polyphenols, tannins, and phenolic terpenes [1]. Indian traditional herbal and Ayurvedic medicine mention many remedies for treating various diseases such as diabetes mellitus, rheumatoid arthritis, kidney, and cardiovascular diseases.

Costus pictus, commonly known as spiral ginger, belongs to the family *Zingiberaceae*. The leaf extract of *C. pictus* contains bioactive compounds such as flavonoid, phenolic compound, tannin, triterpenes, sterols, alkaloids, and vitamins. The extract may serve as a lead medicinal plant to synthesize various semi-synthetic drugs to treat various life-threatening diseases such as diabetes and cancer. Antioxidant activity of *C. pictus* leaf extract was previously reported [16]. The present study is to assess the effect of different extracts of *C. pictus* leaf in quenching free radicals, especially singlet oxygen [4].

METHODS**Plant material**

C. pictus, namely the insulin plant belonging to the family *Zingiberaceae*, leaves were collected, cleansed, shade dried, and powdered using a commercial grinder. The powdered material is kept in a moisture free dry container for further use.

Preparation of plant extract

Solvent from low polar to high polar nature was selected to extract all the lead molecules present in the plant. About 100 g of dried leaf powder was extracted sequentially using hexane, dichloromethane,

ethyl acetate, and methanol according to their boiling point and concentrated using reduced pressure under vacuum. The extracts collected were stored separately in a container.

Drugs and chemicals

The chemicals and solvents utilized for the study were obtained from Sigma-Aldrich and were of analytical grade.

Antioxidant evaluation

Superoxide radical scavenging activity

Superoxide generation in the laboratory is done using 3.0 ml of Tris-HCL buffer with 16 mM concentration containing 0.5 ml of 0.3 mM nitro blue tetrazolium (NBT) and 0.5 ml nicotinamide adenine dinucleotide hydrogen (NADH) solution. About 1.0 ml of various extracts at different concentration (50, 100, 150, 200, and 250 µg/ml) was added with the above mixture. The reaction was initiated by adding 0.5 ml 0.12 mM phenazine methosulfate (PMS) solution to the mixture and incubated at 25°C for 5 minutes. The absorbance was measured at 560 nm against a blank sample. The descending pattern of absorbance at 560 nm with potent antioxidant property thus indicates the consumption of superoxide anion present in the reaction mixture [12]. The percentage inhibition was calculated using the following equation:

$$\text{Superoxide radical scavenging activity} = \{(A_0 - A_1) / A_0\} * 100\}$$

Where, A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates and ascorbic acid was used as reference standard.

Hydrogen peroxide radical scavenging (H_2O_2) assay

A solution of hydrogen peroxide at a concentration of 40 mM is prepared in phosphate buffer extracts (50, 100, 150, 200, and 250 µg/ml) in distilled water is added to hydrogen peroxide solution and absorbance at 230 nm is determined after 10 minutes incubation. Phosphate buffer without hydrogen peroxide served as blank solution [12]. The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\% \text{ Scavenged } (H_2O_2) = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of control and A_1 is the absorbance sample.

Nitric oxide (NO) radical scavenging assay

Griess reagent was freshly prepared by mixing equal volume of 1% sulfanilamide in 2.5% phosphoric acid and 0.1% **naphthylethylenediamine dihydrochloride** in 2.5% phosphoric acid. NO radical quenching was envisaged by adding 0.5 ml of 10 mM sodium nitroprusside in phosphate-buffered saline which was mixed with 1 ml of the different concentrations of the extracts (50, 100, 150, 200, and 250 µg/ml) and incubated at 25°C for 180 minutes. The total volume of the extract mixture was calculated and mixed with an equal volume of freshly prepared Griess reagent. Control was prepared in a similar way done for the test samples but without the extract solution. A volume of 150 µl of the reaction mixture was transferred to a 96-well plate and the absorbance was read at 550 nm. *Butylated hydroxytoluene* (BHT) is used as a positive control.

$$\% \text{ NO scavenging} = \{(A_0 - A_1) / A_0\} * 100\}$$

Where, A_0 is the absorbance of control and A_1 is the absorbance sample.

Statistical analysis

Singlet free radical-like oxygen scavenging such as hydrogen peroxide scavenging activity, superoxide radical activity, and NO scavenging activity were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS software for windows. Estimates of mean, standard error, and significance for aforesaid parameters were calculated.

RESULTS AND DISCUSSION

Antioxidant evaluation

Various free radical scavenging and lipid peroxidation assays were carried out for all the extracts of *C. pictus* from low polar to high polar solvent extraction technique. All reactions were carried out in triplicates and mean value is recorded.

Superoxide radical scavenging activity

The regular metabolic process generates numerous superoxide radicals which are reactive. To establish a biological platform, the PMS-NADH-NBT system was employed to depict a similar situation. Superoxide anion was derived from PMS-NADH coupling reaction which in turn reduces NBT. The consumption of the available superoxide was evaluated by the decrease in absorbance at 560 nm by the efficient extract serving as an antioxidant [8,14]. Methanolic extract of the plant showed a higher % inhibition of 86% at the highest concentration of 250 µg. This was in par with the positive control ascorbic acid. Further analyzing the efficacy, we found that the lower concentrations (50 and 100 µg) of the methanolic extract evidenced a % inhibition which was significantly different from the positive control ($p < 0.05$) (Table 1).

Hydrogen peroxide radical scavenging (H_2O_2) assay

Environment availability of hydrogen peroxide occurs at low concentration levels in all biological system. The leaf crop exposed to heavy pesticide holds minimal amount of the compound where human beings are exposed to H_2O_2 indirectly by its intake. During a metabolic process, H_2O_2 is readily decomposed into singlet oxygen and water. Fenton's reaction leads to the production of hydroxyl radical which is highly reactive [14]. Methanolic extract of the plant showed a higher % inhibition of 94% at the highest concentration of 250 µg. This was in par with the positive control ascorbic acid. Further analyzing the efficacy, we found that the lower concentrations (50 and 100 µg) of the methanolic extract evidenced a % inhibition which was significantly different from the positive control ($p < 0.05$) (Table 2).

NO radical scavenging assay

The metabolic conversion of arginine to citrulline with the formation of NO. These are carried out by specific NO synthases in various cells such as fibroblast and endothelial cells. Nitrate and nitrite are known to be the by-products of decomposition of the compound sodium nitroprusside at a pH of 7.2 [18]. These entities can be quantified using Griess reagent [8]. Methanolic extract of the plant showed a higher % inhibition of 86% at the highest concentration of 250 µg. This was accountable with the positive control BHT. Further analyzing the efficacy, we found that at all concentrations (50-250 µg) of the methanolic extract evidenced non-significant difference with the positive control (Table 3).

DISCUSSION

Dietary supplements and daily food intake may serve as a second-level antioxidant system or as an oxidant scavenger. Flavonoids, carotenoids, alkaloids, and various other phytochemicals present in the diet may otherwise serve as scavenging molecule. The first-level antioxidants present in the system may fail to perform the reduction process due to the prevalence of pathophysiological conditions in the body. Therefore, an external switch over to antioxidant becomes a demand. The reduction process could be of any type, like scavenging, transition metal chelation reduction, etc. A good antioxidant must perform all the activities to restore the regular homeostasis and prevent the oxidation chain reaction. In our study, we have performed three different scavenging techniques such as superoxide, hydrogen peroxide, and NO scavenging technique which will help us to analyze the efficacy of various plant extracts against oxidative stress.

Superoxide anion is not a strong oxidant entity but decomposes to form reactive singlet and hydroxyl radical. These reactive molecules

Table 1: % Inhibition of superoxide peroxide radicals, each value represents average of three analysis (mean±SD)

Concentration in µg	Hexane	Dichloromethane	Ethyl acetate	Methanol	Ascorbic acid
50	23±1.23	26±1.56	34±0.94	45±1.36	67±1.04
100	36±0.95	33±0.78	47±1.23	38±1.78	75±1.67
150	41±0.86	38±1.43	48±0.86	65±1.94	82±1.78
200	45±1.45	43±1.23	67±1.78	82±0.34	85±1.44
250	48±0.67	46±0.84	85±0.57	86±1.23	90±1.87

SD: Standard deviation

Table 2: % Inhibition of hydrogen peroxide radicals, each value represents average of three analysis (mean±SD)

Concentration in µg	Hexane	Dichloromethane	Ethyl acetate	Methanol	Ascorbic acid
50	25±0.96	16±0.87	56±1.56	44±0.96	75±0.92
100	28±1.67	18±0.54	58±1.67	53±0.87	86±0.56
150	34±0.85	22±1.56	66±0.96	76±1.67	86±1.45
200	37±1.76	27±1.67	75±0.78	85±0.96	88±1.67
250	45±1.33	33±1.78	83±1.56	94±0.34	94±1.34

SD: Standard deviation

Table 3: % Inhibition of nitric oxide, each value represents average of three analysis (mean±SD)

Concentration in µg	Hexane	Dichloromethane	Ethyl acetate	Methanol	BHT
50	38±2.55	33±1.45	44±1.45	50±1.67	65±1.76
100	40±1.67	45±1.87	48±1.68	66±1.04	75±1.54
150	46±0.77	47±1.44	67±0.85	72±1.56	77±0.97
200	52±0.86	50±1.67	78±0.95	75±1.62	82±0.56
250	58±0.95	55±0.88	83±1.69	86±1.87	89±0.76

SD: Standard deviation

could be a causative to oxidative stress. The singlet oxygen could react with certain amines and damage the DNA, and the hydroxyl radical has the capability to reduce hemeprotein and cause a reduction in oxygen supply. The superoxide anion assay is done by PMS/NADH/NBT system which is determined by reduction in absorbance at 560 nm. At a highest concentration of the methanolic extract (250 µg), there was about 86% inhibition which was par with the positive control. Thus, the reduction in the absorbance directly reflects the consumption of superoxide anions.

The daily intake of leaf crops which are exposed to harmful insecticides and pesticides may lead to an absorption of 0.28 mg/kg/day of hydrogen peroxide. This could reach the system through inhaling the vapor or mist or through direct eye and skin contact. Hydrogen peroxide could rapidly decompose to oxygen and water with hydroxyl radical formation. This molecule is highly reactive and could cause lipid peroxidation [5]. The methanolic extract of *C. pictus* has shown a significant inhibition at 250 µg which was significantly similar to that of the positive control.

In a biological system, NO is formed by the metabolism of arginine to citrulline by NO synthase with the release of NO. In our experiment, the NO is produced from nitroprusside which actively decomposes to NO in aqueous system and in an aerobic condition leads to the formation of nitrite and nitrate which is determined using Griess reagent. The reduction in absorbance indirectly implies the decomposition of NO which was maximum at 250 µg of the methanolic extract.

CONCLUSION

This research article is focused on *in vitro* methods of antioxidant evaluation. Methanol as a solvent has priority for extraction of plants for evaluating their antioxidant activity. The presence of secondary metabolites such as flavonoids, polyphenols, tannins, and phenolic terpenes may be responsible for the O₂, H₂O₂, and NO scavenging activity. Further investigations are required to find the active component of the active extract and to confirm the mechanism of action. This article could also be a comprehensive ready reference for those who are interested on antioxidant study.

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