WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 6, 475-485. Research Article ISSN 2277 – 7105

# COMPARATIVE ENZYME ASSAY OF *CARISSA CARANDAS* FRUIT AT VARIOUS STAGES OF GROWTH WITH STORED RIPE STAGE

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Article Received on
16 June 2014,

Revised on 11 July 2014,
Accepted on 06 August 2014

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## ABSTRACT

*Carissa Carandas* Linn. (Karaunda) is a widely used medicinal plant by tribals throughout India and popular in various indigenous system of medicine like Ayurveda, Unani and Homoeopathy. All parts of the plant are used in traditional medicine. Traditionally the plant has been used in the treatment of scabies, intestinal worms, pruritus, biliousness and also used as antiscorbutic, anthelmintic. The notable biological activities reported are analgesic, anti inflammatory, anti pyretic, cardiotonic and histamine releasing. Hydrogen peroxide is disposed by catalases and peroxidases. In plants, catalase is found predominantly in peroxisomes (and also in glyoxysomes) where it functions chiefly to remove the $H_2O_2$ formed during photorespiration (or during β-oxidation of fatty acids in glyoxsomes) (Bowler *et al.*, 1992). In spite of its restricted location it may play a significant role in defense against oxidative stress since $H_2O_2$ can readily diffuse across membranes. Some of these enzymes have broad substrate specificity while others can only function with one. The peroxidases with broad specificity are often found in cell wall where they utilize $H_2O_2$ to generate phenoxy compounds that then polymerize to produce components such as lignin (Greppin *et al.*, 1986). In addition to their role in the biosynthesis of cellular components, reactive oxygen species are thought to act as secondary messengers in cells. The present study was formulated to assess the enzyme assay of *C. carandas.* Stored Carissa fruits have specific peroxidase enzyme activities of 0.012units/min/mg protein i.e. decreased almost 2 times then at ripened stage and specific catalase enzyme activities of 0.051units/min/mg protein i.e. increased almost 5 times then at ripened stage and 3 times then at matured stage. The research on pharmaceuticals from Carissa must gain momentum to promote the fruits as functional food and to develop cheap, eco-friendly and safe

therapeutics. The findings reported here are expected to stimulate interest and open the possibility of clinically effective drugs from this genus.

**KEY WORDS:** *Carissa Carandas,* Antiscorbutic, Anthelmintic, Catalases and Peroxidases.

## INTRODUCTION

The plant of *Carissa carandas* Ass. Koroja tenga is an evergreen spiny small shrub with light-grey or yellowish-brown bark which is rough and scaly belonging to the family Apocynaceae. Leaves are coriaceous, elliptic-oblong or ovate-oblong and glabrous measuring 2–7 X 1-5 cm. The fruits are berries measures 1–1.4 cm long, ovoid-oblong or ellipsoidal in shape possessing deep-purple or nearly black when ripe. Ripe fruits are sweet acidic in taste, eaten row or made into jam and jelly, bearing the richest source of iron containing & good content of vitamin C(A.C. Dutta, 1985). *Carissa carandas* is very useful to cure anemia, its fruits have anti-scorbutic properties also. Unripe fruits are astringent, useful in bilious complaints .Flowering occurs in between February to June and fruit formation takes place between April and June (Shah, 2006). The peel and pulp are usually green when immature, but turns dark purple or nearly black in colour when the fruit ripens (Patel, 2009). Peroxidase (POD) includes in its widest sense a group of specific enzymes such as NAD-peroxidase, NADP-Peroxidase, fatty acid peroxidase etc. as well as a group of very non-specific enzymes from different sources which are simply known as POD.It catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc. POD occurs in animals, higher plants and other organisms. Hydrogen peroxide is disposed by catalases and peroxidases. In plants, catalase is found predominantly in peroxisomes (and also in glyoxysomes) where it functions chiefly to remove the $H_2O_2$ formed during photorespiration (or during β-oxidation of fatty acids in glyoxsomes) (Bowler *et al.,* 1992). In spite of its restricted location it may play a significant role in defense against oxidative stress since $H_2O_2$ can readily diffuse across membranes. Some of these enzymes have broad substrate specificity while others can only function with one. The peroxidases with broad specificity are often found in cell wall where they utilize $H_2O_2$ to generate phenoxy compounds that then polymerize to produce components such as lignin (Greppin *et al.*, 1986). In addition to their role in the biosynthesis of cellular components, reactive oxygen species are thought to act as secondary messengers in cells. Plant peroxidase activity seems to be under the strict control depending on the development stage and the environmental stimulus (Gadea *et al.*, 1999). Ali *et al.* (2005) reported that peroxidase activity increases when higher

temperatures were applied. On the other hand, the activation of peroxidase is correlated to the defense responses of fruit in presence of pathogens (Maksimov *et al.,* 2003). Peroxidase enzymes participate in hormone catabolism, phenol oxidation, polysaccharides and cell wall proteins intercrossing, lignin polymerization, fruit ripening and defense against pathogens. During fruit ripening and particularly during climacteric rise, the activity of peroxidase increases along with the polygalacturonase and cellulose enzymes (Robinson, 1991).Endogenous antioxidant enzymes like super oxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, minerals like Se, Mn, Cu, Zn, and certain vitamins exert synergistic actions in scavenging free radicals. Catalase is an enzyme related to the cellular control. Catalase has a double function as it catalyses decomposition of hydrogen peroxide to give water and oxygen and oxidation of H donors e.g. methanol, formic acid, phenol with the consumption of one mole of peroxide.

## MATERIALS AND METHODS

### Collection of samples

Fully Ripe fruits of *C. carandas* were collected from different provinces in Tezpur during August to September. Fruits were stored at deep freez of 4°C in air tight containers.

The method proposed by (Mallik and Singh, 1980; Putter, 1974) & (Luck, 1974) was adopted for assaying the activity of Peroxidase and Catalase in *C. carandas* .

### Principle

Guaiacol is used as substrate for the assay of peroxidase.

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{POD}} \text{Oxidized guaiacol} + 2H_2O$$

In the presence of the hydrogen donor Guaiacol, peroxidase converts $H_2O_2$ to $H_2O$ and $O_2$. The oxidation of Guaiacol to a coloured product called oxidized guaiacol. The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of oxidized or dehydrogenated guaiacol product is a measure of the peroxidase (POD) activity and can be assayed spectrophotometrically at 436nm.

### Reagents

1. Phosphate Buffer 0.1 M (pH 7.0).
2. 20mM Guaiacol Solution 240 mg guaiacol was dissolved in water and volume made upto 100ml. It can be stored frozen for many months.

3. 12.3Mm (0.042%) Hydrogen peroxide 0.14 ml of 30% $H_2O_2$ diluted to 100ml with water and the extinction of this solution should be 0.485 at 240 nm. Prepared freshly.

**PROCEDURE**

**Preparation of Enzyme Extract**

1g of fresh plant tissue was extracted in 3ml of 0.1 M phosphate buffer pH 7 by grinding with a pre-cooled mortar and pestle. The homogenate was centrifuged at 18,000g at 5°C for 15 min. The supernatant was used as enzyme source within 2-4 h. Stored on ice till the assay was carried out.

**Assay of Peroxidase**

To 3.0 ml phosphate buffer solution, 0.05ml guaiacol solution, 0.1 ml enzyme extract and 0.03ml hydrogen peroxide solution was added in a cuvette. The buffer solution was brought to 25°C before assay and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.03 ml of $H_2O_2$ was added and mixed. The change in absorbance was recorded every 30 seconds upto 3 minutes in a spectrophotometer. If the rate of increase is very high, the assay should be repeated with dilute extracts. A graph was plotted with the increase in absorbance against time. From the linear phase, the change in absorbance was recorded per min. The enzyme activity was expressed per unit time per mg of protein or tissue weight. Water blank was used in the assay.

One unit of peroxidase is defined as the change in absorbance/minute at 436nm and measured time in minutes (t) to increase the absorbance by 0.05 unit.Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of extract is calculated as below:

**Enzyme activity (units/litre) = 3.18 X 0.1 X 1000/6.39 X 1 X $\Delta$t X 0.1**

**= 500/$\Delta$t**

**Principle**

The UV absorption of hydrogen peroxide solution can be measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. From the decrease in absorbance, the enzyme activity can be calculated. But this method is applicable only with enzyme solutions which do not absorb strongly at 230-250nm.

**Reagents**

1. Phosphate buffer: 0.067 M (pH 7.0)

2. Hydrogen peroxide in phosphate buffer

0.16 ml of $H_2O_2$ (10%w/v) diluted to 100ml with phosphate buffer and the absorbance of this solution should be about 0. 5 at 240 nm with 1cm light path. Prepared freshly.

**Procedure**

**Preparation of enzyme extract**

Plant tissue homogenized in a blender with M/150 phosphate buffer (assay buffer diluted 10 times) at 1-4°C .The homogenate was centrifuged at 10,000rpm for 20min.s. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The combined supernatants (sometimes opalescent) were used for the assay.

**Assay of Catalase**

$H_2O_2$-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. If t is greater than 60sec the measurements should be repeated with a more concentrated solution of the sample. A graph was plotted with the decrease in absorbance against time. From the linear phase, the change in absorbance was recorded per min. The enzyme activity was expressed per unit time per mg of protein or tissue weight. The enzyme solution containing $H_2O_2$-free phosphate buffer served as control.

One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units. Since the extinction coefficient of $H_2O_2$ at 240 nm under the conditions specified is 3.45 per micromole per ml, the enzyme activity per litre of extract is calculated as below:

**Enzyme activity (units/ml) = 3.45 X df /t X 0.1**

Where**, df**=dilution factor

**RESULTS AND DISCUSSION**

The present study was formulated to assess the catalase and peroxidase enzyme assay of *C. carandas.* Hydrogen peroxide is disposed by catalases and peroxidases. The specific activity of peroxidase enzyme increases from 0.008 units/min/mg protein at the premature fruit stage

to 0.118 units/min/mg protein at its preripened fruit stage, which shows a remarkable increase of 14 times, but eventually (i.e. during the ripening stage) it decreased to 0.029 units/min/mg protein (Table 1; Fig. 1) (Patel, 2009). Similarly, the specific activity of catalase in the *C. carandus* fruit exhibited inconsistency. The specific activity increased by two fold from young stage (0.007 units/min/mg protein) to the mature stage (0.014 units/min/mg protein), but thereafter the specific activity decreased and remained more or less unchanged until ripening. (Table 1; Fig. 2) (Patel, 2009).

**Fig1: *Carissa caradas* plant**

**Table1: Changes in the specific activity of certain enzymes in the fruit of *Carissa carandus* during its successive stages of growth and ripening.* (Patel, 2009)**

| Enzymes | Stages of fruits | | | | |
|---|---|---|---|---|---|
| | Young | Premature | Mature | Pre ripened | Ripened |
| **Peroxidase** (untis/min/mg protein) | 0.017 ± 0.004b | 0.008 ± 0.001a | 0.008 ± 0.006a | 0.118 ± 0.090d | 0.029 ± 0.001c |
| **Catalase** (units/min/mg protein) | 0.007 ± 0.001b | 0.006 ± 0.0006b | 0.014 ± 0.004c | 0.004 ± 0.001a | 0.009 ± 0.002b |

* Values of means followed by different alphabets are statistically significant according to Duncan's multiple range test (DMRT) at 5 % level. (n = 3)

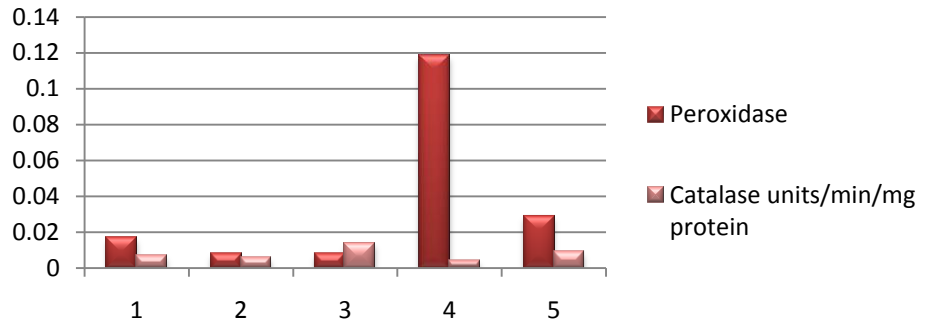


**Fig.2: Changes in the specific activity of certain enzymes in the fruit of *Carissa carandus* during its successive stages of growth and ripening.**

**Table 2: Changes in absorbance of Peroxidase enzyme extract (C*arissa carandas)* with time**

| Sl. No. | Absorbance | Time (s) | Specific ctivity |
|---|---|---|---|
| 1 | 0.057 | 0 | 0.012 |
| 2 | 0.068 | 30 | |
| 3 | 0.069 | 60 | |
| 4 | 0.105 | 90 | |

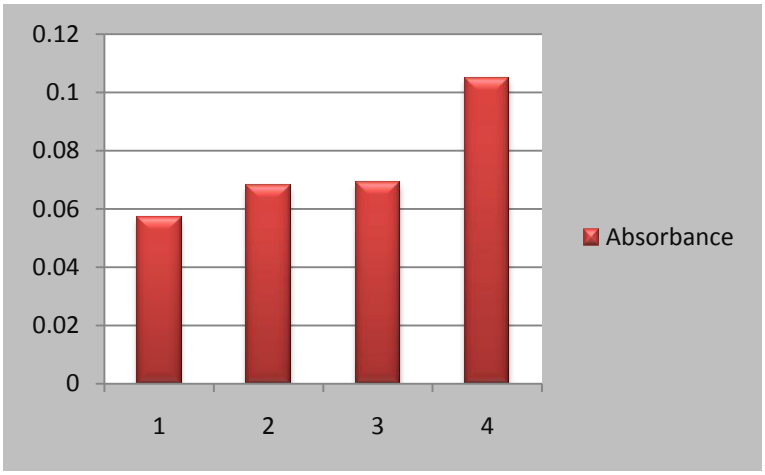


**Fig.3: Changes in absorbance of Peroxidase enzyme extract (C*arissa carandas)* with time**

Most accurate values of enzyme activity are obtained when t is between 1 and 3 min. Time required to increase the absorbance from 0.057 to 0.105 is 1 min. 30 sec i.e. 1.5 min.

Enzyme activity (units/litre) = 500/Δt

= 500/1.5

= 333.33 units/litre

Specific enzyme activity = 0.012 units/min/mg protein

**Table3: Changes in absorbance of Catalase enzyme extract (C*arissa carandas)* with time**

| Sl. No. | Absorbance | Time (s) | Specific activity |
|---|---|---|---|
| 1 | 0.455 | 0 | 0.051 |
| 2 | 0.405 | 30 | |
| 3 | 0.404 | 60 | |

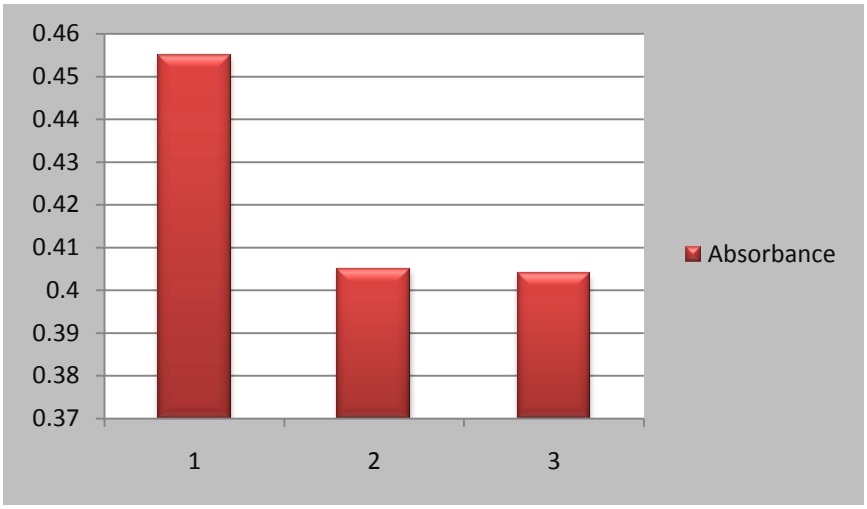


**Fig.4: Changes in absorbance of Catalase enzyme extract (C*arissa carandas)* with time**

Most accurate values of enzyme activity are obtained when t is between 0 to 60 sec. Time required to decrease the absorbance from 0.455 to 0.405 is 30 sec i.e. 0.5 min.

Enzyme activity (units/ml) = 3.45 X df /t X 0.1

= 3.45 X 10/0.5 X 0.1

= 690 units/ml

= 6.9 X $10^5$ units/l

Specific enzyme activity = 0.051units/min/mg protein

Present studies shows at 4°C, stored Carissa fruits have specific peroxidase enzyme activities of 0.012units/min/mg protein i.e. decreased almost 2 times then at ripened stage (Table 2; Fig. 3)and have catalase specific enzyme activities of 0.051units/min/mg protein i.e. increased almost 5 times then at matured stage and 3 times then at matured stage (Table 3; Fig.4).

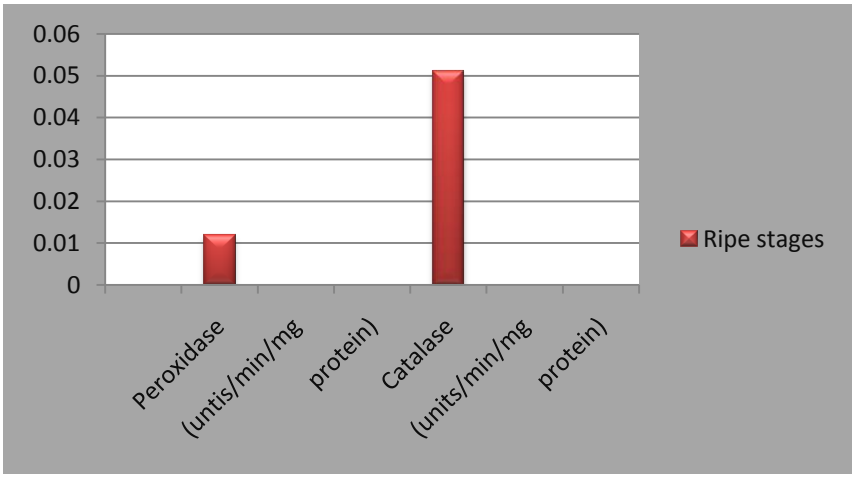


**Fig.5: Changes in the specific activity of peroxidase and catalase enzymes in the fruit of *Carissa carandus* during its stored ripe stage of growth.**

## CONCLUSION

Medicinal plants are sources of important therapeutic aids for alleviating human ailments (Ingale, 2006). The efficacy and safety of medicinal plants naturally represent the object of interest for the pharmacologist and this aspect gives the most important information on herbal medicines (Calapai and Caputi, 2007). Herbal medicine is the use of medicinal plants for the prevention and treatment of diseases; it ranges from traditional and popular medicines of every country to the use of standardized herbal extracts (Firenzuoli and Gori, 2007). There is a growing interest in identifying natural oxidants of herbal resources (Young and Woodside, 2008). One such medicinal plant is *Carissa carandas*, which has been widely used in folklore medicine. Wild fruits present rich sources of antioxidants, with high levels of phenolic compounds, anti-oxidant activities and sugars. Therefore, utilising wild C.carandas fruit as sources of phytochemicals and sugars could offer enormous opportunities for the functional food industry. Fruits extract showed the analgesic, anti-inflammatory and lipase activity. One of the most important considerations in the world today is increasing the production of healthy and nutritious food to feed the burgeoning population on the planet. Hence, the current scenario emphasizes the need to improve resource The antioxidant enzyme Peroxidase exhibited inconsistency in the fruits of *Carissa carandus* (Patel,2009) and in stored fruits (at 4°C) the activity of enzyme further decreased to 0.012units/min/mg protein.

Moreover, this study also opens new vistas by exploring the nutritional value of this fruits. Besides, this study provides new horizon to explore many more underutilized fruits, as they have never been explored or utilized due to lack of awareness in the research community and hence remained underutilized. Moreover, the use of these underutilized fruits can be a substitute to make human diets more balanced and they may play an important role in increasing the production of fruits by introducing these underutilized fruits into commercial market.This study testifies that this plant is an integral part of survival in several developing countries providing the poor population with food and medication. Due to deforestation, overgrazing, encroachments and urbanization, several medicinal plant species are on the verge of extinction. Due to scanty literature and scarce research on this medicinally valuable genus, people are unaware of its multi-pronged potential. The research on pharmaceuticals from Carissa must gain momentum to promote the fruits as functional food and to develop cheap, eco-friendly and safe therapeutics. No doubt, this genus possesses a wealth of prospects to contribute to the field of functional foods and phytomedicines. The findings reported here are expected to stimulate interest and open the possibility of clinically effective drugs from this genus.

**ACKNOWLEDGEMENT**

The authors gratefully acknowledge the DBT sponsored Institutional Biotech Hub and Star College Scheme, for providing us the facilities to perform the research work. The authors are also thankful to Principal, Darrang College, Tezpur for giving permission to perform the work.

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