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ORIGINAL PAPER

Erythrocyte Protoporphyrin Fluorescence as a Biomarker to Monitor the Anticancer Effect of *Semecarpus Anacardium* in DMBA Induced Mammary Carcinoma Rat Model

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Abstract Endogenous fluorescence has been proposed as a means of aiding the diagnosis of various malignancies. It has been suggested that erythrocytes may be the carriers of fluorophors that accumulate in cancer tissue and may be useful in the diagnosis and treatment of malignancies. Hence, the present study was designed to explore the spectrofluorimetric analysis of blood components as a marker for the analysis of mammary carcinoma treatment and also to bring about the protective effect of the drug *Semecarpus anacardium* on oxidative stress mediated damage of erythrocytes. Fluorescence spectra of the blood components were studied and also the level of lipid per oxides and antioxidant enzymes status in erythrocytes were determined in DMBA induced mammary carcinoma rats treated with *Semecarpus anacardium* Linn nut milk extract. Fluorescence emission spectroscopy of blood components are altered under cancer conditions and the drug effectively ameliorated these alterations in mammary carcinoma induced rats. The drug also effectively reduced the oxidative stress induced erythrocyte damage thereby restoring the erythrocytes antioxidant status. These results suggest that erythrocytes may be the carriers of fluorophors that accumulate in cancer tissue and hence acts as new biomarkers for the diagnosis and treatment.

Keywords Breast cancer · Fluorescence spectroscopy · Erythrocytes · Protoporphyrin · Antioxidants

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Introduction

Breast cancer accounts for 23 % of all newly occurring cancers in women worldwide and represents 13.7 % of all cancer deaths [1, 2]. It is the most frequent cancer in both developed and developing regions (estimated 690,000 new cases in each region) as well as the most frequent cause of cancer death in these regions (280,000 deaths in developing countries) of the world [1, 2]. In India, breast cancer is the second most common cancer (after cervical cancer) with an estimated 115,251 new diagnoses and the second most common cause of cancer-related deaths with 53,592 breast cancer deaths in 2008 [1]. The age-standardised incidence rate for breast cancer in India is 22.9 per 100,000, one-third that of Western countries and the mortality rates are disproportionately higher [3, 4].

Although Tamoxifen is able to reduce the risk of recurrence in localized breast cancer, there is still no definitive way to prevent breast cancer [5]. Therefore, identification of new and efficient anticancer drugs has always been a focal point in cancer research. Nowadays, more effort has been put forward to study the curative effect of traditional herbal drugs in various pathological conditions.

Semecarpus anacardium Linn (Anacardiaceae) nut milk extract is one such drug which has been used in Siddha system of medicine against various ailments. The chief constituents present in the nut milk extract include trihydroxyflavone, semecarpol, anacardoside and bhilawanols and this has been confirmed through HPLC and HPTLC analysis of both the nut and milk extract [6–9]. Several studies carried out in our laboratory have established the antioxidant, anti diabetic [10], hypolipidemic [11], cardioprotective [12] and also anticancer effects against mammary carcinoma [13] and chronic myeloid leukemia [14].

Fluorescence spectroscopy has gained increasing importance for the development of protocols for early diagnosis in

various types of organ disease because of its high sensitivity to alterations in the function, morphology and microenvironment in cells and tissues [15]. Endogenous fluorescence has been proposed as a means of aiding the diagnosis of various malignancies by studying the pathological changes that occur [16] during malignancy. There are a number of detailed studies of tissue characterization using endogenous fluorophores as tumor markers in fluorescence spectroscopy [17, 18] but only limited reports on using body fluids to diagnose cancer are available. It has been suggested that erythrocytes may be the carriers of fluorophors that accumulate in cancer tissue and may be useful in the diagnosis and treatment of malignancies [19].

The erythrocytes are particularly vulnerable to oxidative damage due to continuous exposure to high oxygen tension as well as the presence of large amount of iron, a potent catalyst for oxygen free radical production and poly unsaturated fatty acids (PUFA) major source for per oxidation [20]. The normal erythrocyte is however resistant to oxidative damage because it is rich in antioxidant enzymes. The plasma is also richly endowed with aqueous radical trapping antioxidants [21]. Low levels of essential antioxidants in circulation have been associated with an increased risk of cancer [22].

Hence, with this background the present study was designed to study the fluorescence of blood samples in mammary carcinoma bearing animals to discriminate them from their respective normals using fluorescent spectroscopy and also to bring about the protective effect of the drug *Semecarpus anacardium* on ameleiorating these changes by minimising the oxidative stress induced eythrocyte membrane damage.

Materials and Methods

Animals and Diet

Adult female albino rats of Sprague–Dawley strain weighing 180±10 g were purchased from King Institute of Preventive medicine, Chennai, India. The rats were fed with a commercial rat feed and were accommodated in well ventilated spacious cages. Food and water were given ad libitum. The animals were maintained under standard conditions of humidity, temperature (27 °C) and light (12 h light/dark). Animal experimentation was conducted according to the institutional regulations. IAEC No: 02/081/06

Drugs and Chemicals

Semecarpus anacardium nut milk extract was prepared according to the formulary of Siddha medicine [23]. 7, 12-dimethyl benz[a]anthracene (DMBA) was obtained from Sigma Chemicals, St. Louis, Mo., U.S.A. All other chemicals and solvents used for the study were of analytical grade.

Experimental Design

The animals were randomly divided into three groups of six animals each. Group I: Control animals, Group II: Breast cancer was induced in overnight-fasted animals by a single dose of DMBA in olive oil (25 mg/kg body weight) [24, 25] by gastric intubation. Group III: Breast cancer-induced animals (as in Group II) were treated with *Semecarpus anacardium* Linn nut milk extract (200 mg/kg body weight/day) in olive oil orally by gastric intubation for 14 days.

Experimental Procedure

Body weight changes were recorded at weekly intervals. After the experimental period, the overnight fasted animals were sacrificed by cervical decapitation. Blood was collected with EDTA as anticoagulant. Plasma was separated by centrifugation at 2000*g* for 20 min.

Biochemical Assays

Preparation of hemolysate and isolation of erythrocyte membrane was carried out by the method of Dodge et al.[26]. Lipid peroxide concentration was determined by thiobarbituric acid reaction as described by Cynamon et al. [27], Superoxide dismutase (SOD) was determined by the method of Marklund and Marklund [28]. Catalase (CAT) and glutathione peroxidase (GPx) activities were estimated by the method of Sinha [29] and Rotruck et al. [30], respectively. During the experimental period i.e., before the animals were sacrificed, the animals were weighed, explored by inspection and palpation and the two major and perpendicular diameters of each tumor were measured with a caliper. From these data, latency time and tumor volume were studied. Latency time was analyzed by means of the average time for to first tumor development. Total tumor volume was measured as described by Escrich et al.[31] $v=4/3\pi\,(d_{1/2})\times(d_{2/2})^2$, where d_1 and d_2 are the two diameter of the tumor ($d1>d2$). At sacrifice, the volume of each tumor calculated using its three diameters: $v=4/3\pi\,(d_{1/2})\times(d_{2/2})\times(d_{3/2})$;$(d1>d_2>d_3)$.

Histopathological Analysis

Formalin-fixed mammary tumour samples were paraffin embedded, sectioned (3 mm thickness) and placed on glass slides. Paraffin-embedded sections of tissue were deparaffinized, rehydrated with graded alcohol and stained with Harris' haematoxylin and eosin (Dako, Glostrup, Denmark) in a Leica Autostainer (Wetzlar, Germany). Histopathological evaluation was performed according to Costa et al. [32].

Spectrofluorometric Analysis

To the known volume of supernatant (hemolysate), pale yellow to white pellet (erythrocyte membrane), plasma and erythrocyte, 2 ml of analytical grade acetone was added. This was vortexed and centrifuged at 5000 rpm for 10 min. The clear supernatant (acetone extracts) were subjected to fluorescent spectral analysis at 400 nm excitation using a spectrofluorometer (Kontron, SFM25, USA) and the emission spectra were scanned from 430 nm to 700 nm.

Statistical Analysis

Results were represented as mean±S.E.M. of six rats. The results were computed statistically (SPSS software package, version 7.5) using One-way Analysis of Variance (ANOVA). Post hoc testing was performed for intergroup comparison using Student – Newman – Keul multiple comparison test. Values of $p<0.001$ were considered significant.

Results

Effect of *Semecarpus anacardium* on Body Weight Changes

Figure 1 represents the body weight changes of the control and experimental group of rats. Body weights were recorded from the day of tumour induction, till the completion of the experimental period. Initial and final body weights were plotted. Initially there was no significant change in the body weight of the control and experimental animals. The control (group I) animals added on weight steadily throughout the experimental period, whereas the growth rate of mammary carcinoma bearing (group II) animals were significantly ($p<0.05$) reduced when compared with control group of rats.

Effect of *Semecarpus anacardium* on Tumor Weight Changes

Figure 2 represents the tumor volume of the treated and untreated animals. Tumor volume and survival times have been used extensively to measure the anticancer activity of synthetic or natural products. The tumor volume of Group III rats was significantly reduced as compared to group II rats indicating the tumor growth inhibitory potential of the drug.

Effect of *Semecarpus anacardium* on Erythrocyte Membrane Lipid Peroxidation and Antioxidants Enzyme Activity

Table 1 shows the level of lipid peroxides (LPO) in plasma and erythrocyte membrane of control and experimental animals. The levels of lipid peroxides in plasma ($p<0.001$) and erythrocyte membrane ($p<0.01$) of mammary carcinoma bearing rats were found to be significantly increased when compared to control rats. Drug treated animals showed a significant decrase in the levels of lipid peroxides when compared with mammary carcinoma bearing rats.

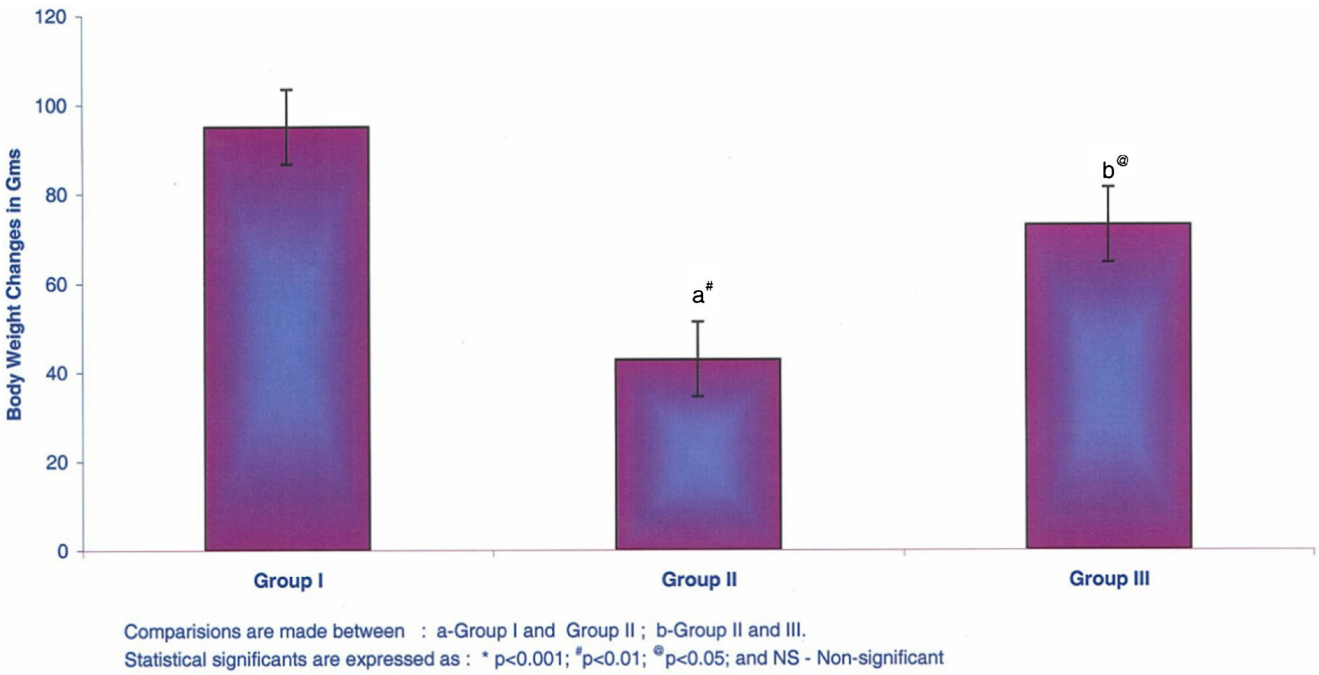

Fig. 1 Effect of *Semecarpus anacardium* on body weight changes

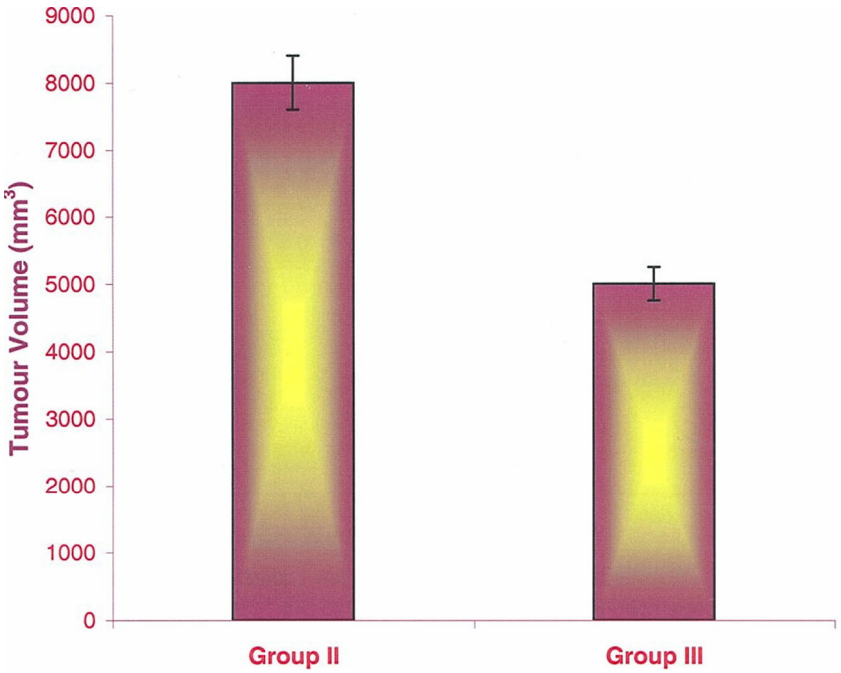

Fig. 2 Effect of *Semecarpus anacardium* on tumor volume

Table 2 shows the levels of activities of enzymic antioxidants viz; superoxide dismutase, catalase and glutathione peroxidase in hemolysate of the control and experimental group of rats. The activity of enzymic antioxidants were found to be significantly ($p<0.001$) decreased in mammary carcinoma bearing rats when compared to control rats. On drug treatment, the activities of antioxidants, SOD($p<0.05$); CAT($p<0.001$) and GPx ($p<0.001$) were significantly increased when compared to induced group of rats.

Effect of *Semecarpus anacardium* on Spectral Analysis of Erythrocyte Membrane

The fluorescent spectra and their emission characteristics were given in Fig. 3 and Table 3 respectively. The plasma and erythrocyte of normal and treated rats showed a prominent peak at 430 nm and decreases at longer wavelengths as shown in Fig. 3. The erythrocyte membrane of control, cancer and treated samples showed a maximum intensity at 440 nm and a second peak at 630 nm while erythrocyte membrane of the cancer sample in addition showed a secondary peak at 540 nm. The cancer plasma and erythrocytes showed a peak at 630 nm.

Effect of *Semecarpus anacardium* on Histopathological Changes in Breast Tissue

Tissue section from Group I rat (Fig. 4a) depicts the normal architecture of the breast tissue. Tissue sections from Group II cancer bearing rats (Fig. 4b) showed the tumour composed of sheets and nests of cells with vesicular nuclei and indistinct cytoplasmic margins. Tubular formations are seen within these nests of cells. Pleomorphism and marked increase in mitotic activity were observed.

In Group III (Fig. 4c) lobules of normal breast tissue are seen within the subcutaneous fat. The histopathological changes observed indicate that the drug has inhibitory effect on tumour progression.

Table 1 Effect of *Semecarpus anacardium* on levels of lipid peroxides in control and experimental animals

Parameters	Group I	Group II	Group III
Plama (n moles MDA formed / min / mg protein)	3.89±0.12	8.17±0.81[a*]	6.12±0.33 [b*]
Erythrocyte membrane(n moles MDA formed / min / mg protein)	1.75±0.09	2.12±0.19 [a*]	1.81±0.13 [b*]

Values are expressed as mean ± SD for 6 animals

Comparisons are made between a – Group I vs Group II; b- Group II vs Group III

NS non significant

Statistical significance are expressed as * $p<0.001$; #$p<0.01$; @ $p<0.05$

Table 2 Effect of *Semecarpus anacardium* on levels of enzymatic antioxidants in the erythrocyte membrane of control and experimental animals

Parameters	Group I	Group II	Group III
Superoxide Dismutase (unit/ min/mg protein)	2.9±0.27	1.5±0.81[a*]	1.9±0.1 [b*]
Catalase(μmol of H_2O_2 consumed / min / mg Hb)	48±1.3	33±1.3 [a*]	42±1.07 [b*]
Glutathione peroxidase (unit/ min/mg protein)	13±0.09	6±0.04 [a*]	8±0.05 [b*]

Values are expressed as mean ± SD for 6 animals

Comparisons are made between a – Group I vs Group II; b- Group II vs Group III

NS non significant

Statistical significance are expressed as * $p<0.001$; #$p<0.01$; @ $p<0.05$

Discussion

Breast cancer, the most common cancer and a major cause of death in women, makes up about one-tenth of all new cancer diagnoses worldwide [33]. More efforts have been put forward for the identification of newer, affordable and easily assessable markers for the diagnosis and also screening of newer plant derived products in the treatment against cancer. In hematology, cancer diagnosis and screening by application of native fluorescence spectroscopy of porphyrin is gaining importance in recent times. Several studies have shown that there is a relationship between porphyrin (with fluorescence at 630 nm) and cancer cell proliferation in an animal tumor model [34, 35]. With this aim, the present study was designed to bring about the flurorescent emission spectroscopy of blood components and the protective role of the drug on oxidative stress induced damage on the erythrocyte membrane.

The sharp decline in the body weight of mammary carcinoma bearing animals may be due to cancer cachexia which results in progressive loss of body weight which is mainly due to tissue wasting. Cancer patients lose weight both due to fat and muscle mass loss. Muscle loss occurs due to increased amount of alanine originating from muscle degradation and utilisation for gluconeogenesis [36]. Upon drug administration, there was a gradual increase in body weight, which denotes the anti neoplastic activity of the drug.

The tumour volume has been used extensively to measure the anticancer activity of synthetic or natural products. Considerable increase in tumour volume was observed in cancer induced animals whereas in drug treated animals the tumour did not disappear totally but a significant regression was found showing the inhibitory effect of the drug on tumour growth. This might be due to the anticancer effect of the drug which has been known to prevent carcinogenesis in terms of tumour incidence, tumour multiplicity, tumour size and tumour growth kinetics [37].

Reactive oxygen species (ROS) have been implicated in the pathogenesis of various conditions including cancer [38]. By-products of lipid per oxidation have been shown to cause profound alterations in the structural organization and

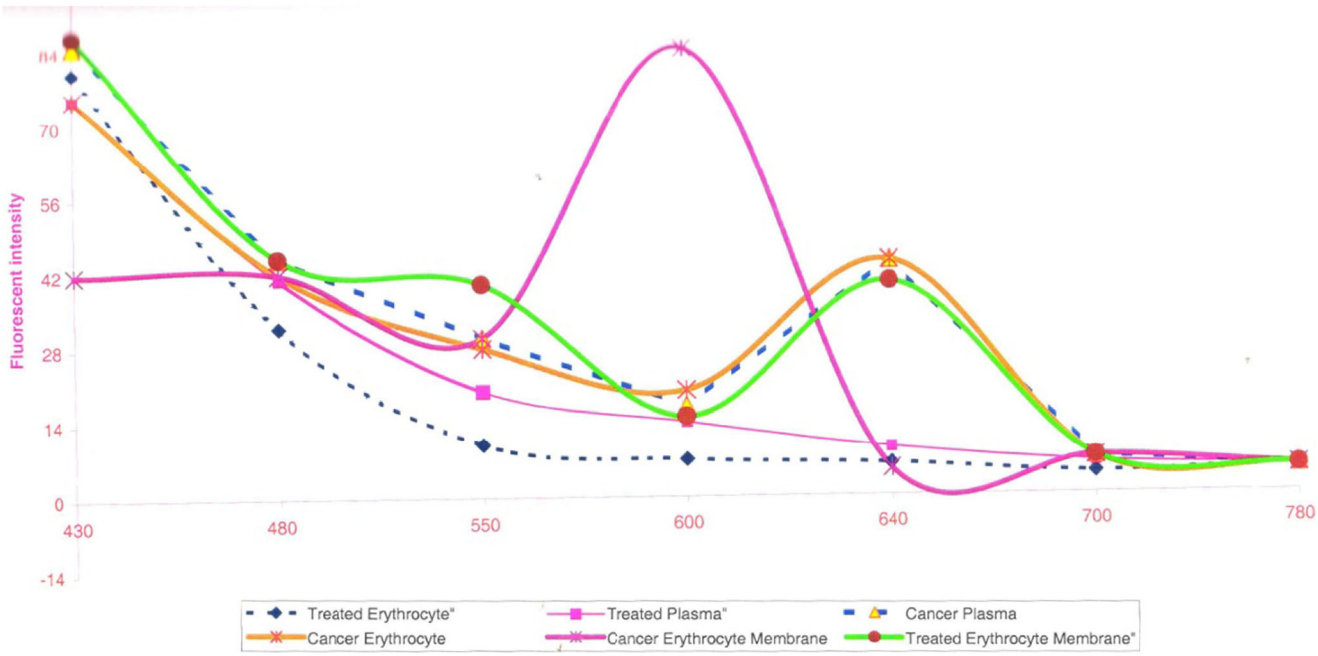

Fig. 3 Effect of *Semecarpus anacardium* on emission spectra of plasma Erythrocyte and Erythrocyte membrane of the experimental animals

Table 3 Emission characteristics of plasma, erythrocyte, erythrocyte membrane of experimental rats excited at 400 nm

Parameters	Group I	Group II	Group III
Plama	4.00±0.02	1.1±0.03^{a*}	3.7±0.02 ^{b*}
Erythrocyte	2.51±0.01	0.3±0.04 ^{a*}	1.9±0.01 ^{b*}
Erythrocyte membrane	2.3±0.04	3.3±0.01 ^{a*}	2.5±0.02 ^{b*}

Values are expressed as mean ± SD for 6 animals

Comparisons are made between a – Group I vs Group II; b- Group II vs Group III

NS non significant

Statistical significance are expressed as *p<0.001; #p<0.01; @ p<0.05

functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids [39]. However, the deleterious effects of reactive oxygen species and lipid peroxides are protected by an array of endogenous antioxidant defense systems, by acting as a potent scavenger of free radicals as well as inhibitors of neoplastic process.

The simplicity, availability and ease of isolation make erythrocyte membrane as an excellent model for membrane studies. Erythrocytes and erythrocyte membrane are more vulnerable to lipid per oxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acid, respectively. On the other hand, the erythrocytes contain multi concentration defense mechanisms against free radical-induced lipid per oxidation, which include both enzymatic and non-enzymatic antioxidants [40].

Increased levels of lipid per oxidation products play a role in the early phases of tumor growth [41]. The significantly increased elevations of MDA levels in mammary carcinoma bearing animals observed in our study are consistent with other studies [42, 43] demonstrating that erythrocyte membranes are more susceptible to oxidative damage. Drug treated animals showed a significant decrease in their levels. This may be due to the free radical quenching effect of the various flavanoids present in the drug *Semecarpus anacardium*. Flavonoids possess free radical quenching activity and protect against lipid per oxidation which reduces lipid per oxidation by enhancing host detoxification system [44].

Antioxidants are the body's first resource for protection against the diverse free radicals and other oxidative factors. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP-x) scavenge free radicals, and they are closely related to the modulation of carcinogenesis.

Superoxide dismutase (SOD) and Catalase (CAT) are the two major enzymes that are directly involved in the elimination of reactive oxygen species. The superoxide dismutase

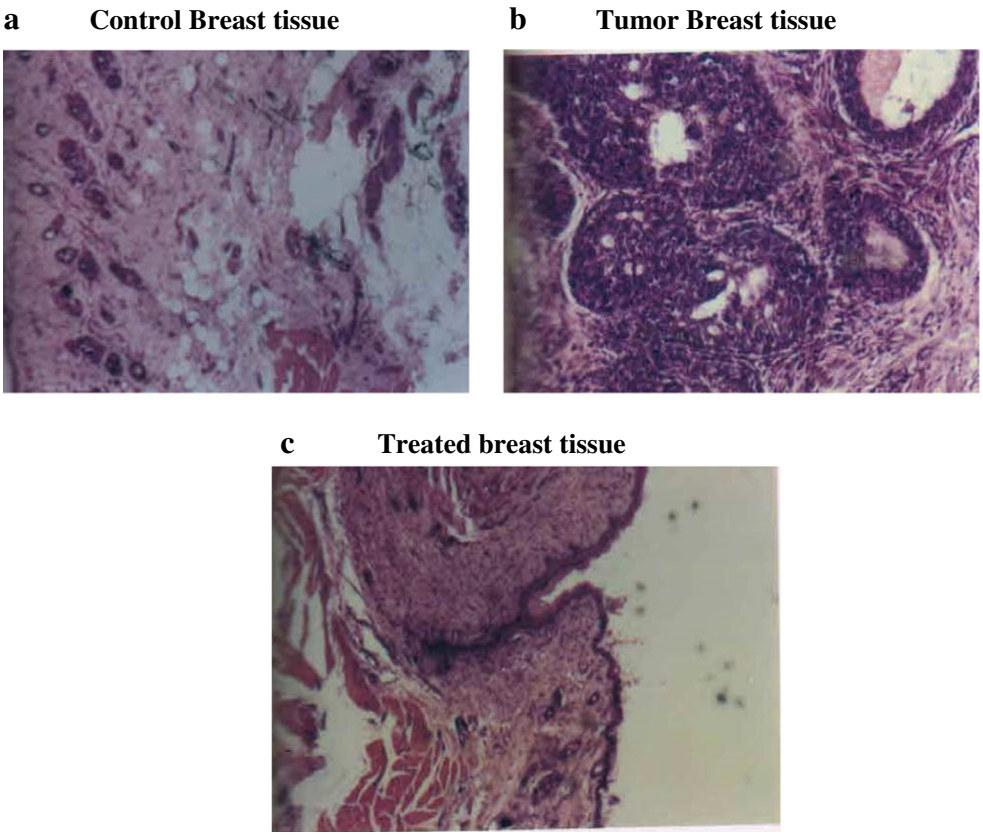

Fig. 4 Effect of *Semecarpus anacardium* on histopathological changes in breast tissue. **a** - Control breast tissue showing normal architecture. **b** - Tumor breast tissue showing pleomorphism with increased mitotic activity. **c** - Treated breast tissue showing lobules of normal breast tissue

enzyme is the foremost important line of antioxidant enzyme defense against reactive oxygen species [45]. It is present in cytosol and mitochondria, which in turn decline the superoxide anion to hydrogen peroxide and water. SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide that damages the membrane and biological structure.

Glutathione peroxidase (Gpx) is a cytoplasmic enzyme that catalyzes the detoxification of hydrogen peroxide to H_2O using the reducing equivalents of glutathione [46]. GPx is a seleno-enzyme which catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. GPx is a selenium containing enzyme present in significant concentrations, detoxifies H2O2 through the oxidation of reduced glutathione [47]. The decreased activity of these enzymes in cancer bearing animals might be due to super saturation of SOD with a high concentration of ROS. The levels of free radicals overcome the saturation level due to increased lipid peroxides. The decline in SOD activity leads to down regulation of H_2O_2. Since H_2O_2 is the substrate for the enzymes CAT and GPx, they were also found to be decreased. Upon treatment with the drug the activity of SOD was restored to near normal level because it produces hydrogen peroxide, which in turn revert the activity of CAT and GPx to near normal levels.

The most commonly used techniques such as mammograms and fine-needle aspiration from the breast for the detection of breast cancer have their own limitations. Hence there is a need for a more sensitive and simpler method of early detection.

Optical spectroscopy provides new ways to characterize physical and chemical changes occurring in tissues and cells and thereby offers exciting possibilities for novel diagnostic and therapeutic approaches [17]. Among the various methods, fluorescence-based techniques are currently of great interest because of their sensitivity to minute variations in the amounts of native fluorophores present in tissues and body fluids. The specific optical spectra of a tissue sample contain information about the biochemical composition and the structure of the tissue both of which undergo a change during malignant transformations. These changes can be detected as an alteration in the fluorescent spectral profile of these tissues and several studies have shown that they can serve as potential biomarkers in the diagnosis and treatment against various types of cancer [19, 48].

Autofluorophors or fluorophors of native tissues are characteristics of a given tissue and any alteration in the pathological status can be sensitively detected using fluorescent spectroscopy. Since it is well known that tumor angiogenesis takes place during the proliferation, it is thought that the transport of these fluorophors may be possible and can be detected in blood erythrocytes. The normal and cancer samples produced their unique behaviour in the emission spectra on excitation at 400 nm.

Flavonoids are reported to protect erythrocyte membrane from the damage by scavenging reactive oxygen species generated in carcinogenesis. The acetone extracts of cancer erythrocytes fluoresce more than its plasma in terms of intensity (FI). This suggests that there is a definite relationship between the erythrocytes and the fluorophores at ~630 nm. In the present study the ratio of fluorescent intensity at 530 nm /630 nm was found to be significantly decreased in plasma and erythrocytes of cancer samples and erythrocyte membranes showed an increase when compared to normal and treated samples. The increase in the ratio of fluorescent intensity at 530 nm/630 nm in erythrocyte membrane and decrased ratio in the plasma and erythrocyte may be due to the alterations in the ratio of flavin/porphyrin contents in the cancer samples (Group II) compared to control (GroupI) and treated samples (Group III).

We found that the ratio of fluorescent intensity at 530 nm/630 nm in cancer plasma and erythrocytes is below one. The 630 nm peak observed in the cancer plasma and erythrocytes suggests that the fluorophors present in the erythrocyte membrane of both samples found to have a close agreement with the fluorophors that accumulated in the cancer tissue which is in accordance with the reports of Balasubramaniam et al.[49] .

Yang et al.[50] reported that porphyrin compounds showed characteristic autofluorescence at 630 nm and 690 nm and suggested that they may be from the degradation of haemoglobin. The degradation of haemoglobin under cancer condition may contribute to the hike in the porphyrin content. Porphyrin is a component of heme derived from intermediate porphyrinogens in the biosynthesis pathway to proto-heme, and it occurs naturally in small amounts in all living cells. Under normal conditions, the synthesis of protoporphyrin is under feedback control; that is, cells produce it at a rate just sufficient to match their heme levels, but with excessive cellular proliferation the feedback mechanism loses control, and the excess porphyrin thus produced appears in the tissues and blood. There are number of porphyrins found in the body, and the red fluorescence associated with cancer is most likely due to protoporphyrin [51]. Protoporphyrin IX (PpIX) accumulates in cancerous tissues as a consequence of tumor-specific metabolic alterations. Our results are in consistent with that of other studies which have used protoporyphyrins in the diagnosis and analysis of cancer severity and treatment [52–54]. These alterations were restored in drug treated group of rats which may be due to the protective effects of various flavonoids present in the drug.

There was also a good correlation between the histological alterations observed in the breast tissue of control and experimental group of rats with the fluorescence spectroscopy data.

Conclusion

The results of the current study indicate that RBC's are more vulnerable to oxidative damage under cancer condition and our drug *Semecarpus anacardium* by means of its increased free radical scavenging effect [55] and antioxidant activity has prevented this damage. These results also suggests fluorescence emission spectroscopy of blood components are altered under cancer conditions and erythrocytes may be the carriers of fluorophors that accumulate in cancer tissue and hence acts as new biomarkers for the diagnosis and treatment of cancer and the drug by means of its anticancer effect has also been able to modulate the emission characteristics of the erythrocyte membrane.

Conflict of Interest The authors declare that there is no conflict of interest among authors.

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