Original Article

A study of the free radical scavenging effects of *Piper betle* leaf extract in patients with vitiligo

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ABSTRACT

Background: Vitiligo is an idiopathic skin disease manifested by depigmented macules. It is characterised by melanocyte destruction, and redox imbalance is proposed to play a contributory role. **Aim:** The aim of this study was to analyze the effects of an ethanolic extract of *Piper betle* leaves on the generation of reactive oxygen species in erythrocytes sourced from vitiligo patients. **Methods:** The effect of *Piper betle* on the generation of reactive oxygen species in erythrocytes was measured by flow cytometry in patients with active and stable vitiligo versus healthy controls, using 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate. **Results:** The generation of reactive oxygen species in erythrocytes was higher in patients with vitiligo (n = 23) compared to healthy controls (n = 18). The geometrical mean fluorescence channel was 23.05 ± 2.11 in patients versus 17.77 ± 1.79 in controls, P = 0.039. The levels of reactive oxygen species were higher in patients with active vitiligo. Treatment of erythrocytes with *Piper betle* in concentrations of 0.5 and 1.0 μg/ml significantly decreased the baseline levels of reactive oxygen species by 31.7% in healthy controls, and 47.6% and 44.3% in patients with active vitiligo, respectively. *Piper betle* effectively scavenged hydrogen peroxide, which was evident by a decrease in the geometrical mean fluorescence channel by 52.4% and 62.9% in healthy controls, and 45.0% and 57.0% in patients with active vitiligo. **Limitations:** The study had a small sample size. Future studies should focus on evaluation of the antioxidant role of *Piper betle* at the lesional site. **Conclusion:** This pilot study indicates that patients with active vitiligo demonstrate enhanced generation of reactive oxygen species in erythrocytes, which was significantly reduced following *ex vivo* treatment with *Piper betle*.

Key words: Anti-oxidant, free radicals, *Piper betle*, reactive oxygen species, vitiligo

INTRODUCTION

Vitiligo is an acquired, idiopathic skin disorder manifested by depigmented macules and patches of varying size and shape.[1] It affects 0.1–2.0% of the population with no age, gender or racial predilection.[2] The prevalence rates are even higher in some parts of India, being 8.8% in Gujarat and Rajasthan.[3] A consistent feature is the loss of functioning melanocytes but the pathogenic contributory mechanisms remain unclear. Several hypotheses have been proposed, and include autoimmune, oxidative stress and neurological mechanisms.[4] However, no single cause can account for the loss of melanocytes, and it is more likely to be a combination of several mechanisms. This is known as the "convergent theory".[4]

A role for oxidative stress as the initial trigger for melanocyte degeneration has been suggested

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as the disease is consistently accompanied by an imbalance in the pro-oxidant/anti-oxidant system.[5,6] Accumulation of pro-oxidants such as hydrogen peroxide, and lowering of anti-oxidant enzymes such as catalase has been recorded in the epidermis and blood.[7,8] Systemic oxidative stress has been demonstrated in active and stable vitiligo. This was more pronounced in active cases where levels of pro-oxidants such as serum malondialdehyde were significantly raised.[9] In active vitiligo, the levels of selenium (present in a family of seleno-enzymes such as glutathione peroxidase and thioredoxin reductase) and superoxide dismutase activity are raised. This suggests that adaptive mechanisms play a role in combating oxidative stress.[9] The role of oxidative stress has also been demonstrated in erythrocytes from patients with vitiligo, being more pronounced in active disease.[10] Interestingly, the generation of reactive oxygen species in unstable vitiligo patients responding to immunosuppressive drugs was comparable to those having stable vitiligo.

Piper betle Linn. (Family: Piperaceae), a perennial climber, is widely recognized as a traditional medicinal plant. The leaves have been demonstrated to have anti-inflammatory, anti-oxidant and radio-protective activities, the key phytoconstituent being allylpyrocatechol.[11-16] Hence, this study was undertaken to evaluate the anti-oxidant potential of an ethanolic extract prepared from the leaves of *Piper betle*, in erythrocytes from patients suffering with vitiligo.

METHODS

Reagents and chemicals

All chemicals used were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA) except 5-(and-6) -chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA, USA). This (1 mM stock) was prepared in dimethylsulfoxide, stored at −20°C and diluted immediately before use.

Preparation of *Piper betle* extract

Fresh leaves of *Piper betle* were obtained locally and authenticated by the Botanical Survey of India (Shibpur, Howrah, India).[11,12] The leaves were air dried, powdered and the ethanolic crude extract was prepared as previously described.[11] The resultant extract was dissolved in propylene glycol and stored at 4°C.

Study population

A cross-sectional, analytical study was carried out in patients with vitiligo. They were recruited from the dermatology outpatient department of the Institute of Post Graduate Medical Education and Research and SSKM Hospital, Kolkata, India. The study protocol was approved by the Institutional Ethics Committee. The sample size was not estimated as this was a pilot study. The study population included patients with vitiligo and healthy individuals. Informed consent was obtained from all patients. They were recruited by the dermatologist based on the history, clinical features and vitiligo disease activity score. They were classified into active and stable disease cases; the criteria for active disease being extension of existing lesions, and/or appearance of new lesions within the last six months.[2] The inclusion criteria were all patients with vitiligo who were above the age of 18 years who had no history of smoking or alcohol intake, and were not on any other medication. Healthy controls (age and gender matched) were recruited from the staff and students at our institute. We excluded patients with other co-existent dermatoses such as psoriasis and atopic dermatitis and patients receiving systemic anti-oxidants or systemic corticosteroids during the previous six months. Heparinized venous blood (2 ml) was collected from the medial cubital vein.

Measurement of reactive oxygen species in erythrocytes

Measurement of reactive oxygen species in erythrocytes was done using 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate. This is a chloromethyl derivative of 2', 7'-dichlorodihydrofluorescein diacetate, which contains an additional thiol reactive chloromethyl group that enhances its ability to bind to intracellular components thereby prolonging the dye's cellular retention. It is a non-fluorescent, lipid soluble, membrane-permeable dye that freely enters the cell and is deacetylated by intracellular esterases to form the hydrophilic, impermeable, non-fluorescent dye 5-(and-6) -chloromethyl-2'-7'-dichlorodihydrofluorescein. This is rapidly oxidized to form the highly fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein. Thus, the level of intracellular reactive oxygen species is directly proportional to the intensity of fluorescence.[17]

Optimization of 5-(and-6)- chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate was done in erythrocytes; briefly, erythrocytes (5×10^5) were incubated for 30 min at 37°C with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydro fluorescein diacetate (0.5–2.5 μM). A concentration of 1 μM was found to be optimal and used throughout the study. Estimation of generation of reactive oxygen species was done within 3 h of collection of blood.

Determination of reactive oxygen species generation in erythrocytes

Estimation of the generation of reactive oxygen species was performed by labeling erythrocytes from patients with vitiligo and healthy controls with 5-(and-6)-chloromethyl -2'-7'-dichlorodihydrofluorescein diacetate (1 μM) for 30 minutes at 37°C, and acquiring the fluorescence with a flow cytometer.

Evaluation of the effect of *Piper betle* on the generation of reactive oxygen species in erythrocytes

Erythrocytes were pretreated with *Piper betle* (0.5 and 1.0 μg/ml, 1 hour, 37°C) and subsequently labeled with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (1 μM, 30 minutes, 37°C). The resultant fluorescence was acquired and analyzed in a flow cytometer.

Effect of *Piper betle* on scavenging of reactive oxygen species in erythrocytes

For evaluation of the free radical scavenging effect, erythrocytes were pretreated with *Piper betle* (0.5 and 1.0 μg/ml, 1 hour, 37°C) followed by hydrogen peroxide (0.5 mM, 1 hour, 37°C) and after labeling them with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, the fluorescence was acquired and analyzed in a flow cytometer.

Flow cytometry

Erythrocytes were monitored for their intracellular fluorescence on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA), equipped with an argon-ion laser (15 mW) tuned to 488 nm. The fluorescence of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein was collected in the FL1 channel, equipped with a 530/30 nm band pass filter. The cells were gated on the basis of the characteristic morphology of erythrocytes, i.e. forward scatter and side scatter properties. Fluorescence was acquired in the log mode and expressed as a geometrical mean fluorescence channel, or the central tendency of fluorescence in the analyzed cells. A total of 10,000 gated events were acquired. The data was analyzed using the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data was analyzed using GraphPad Prism software version 5.0 (Graph Pad Software Inc., La Jolla, CA, USA) and IBM SPSS Statistics 20 (IBM Corporation, New York, USA) where $P < 0.05$ was considered statistically significant. Demographic and disease activity parameters were summarized using descriptive statistics. The data was expressed as median and interquartile range, mean ± standard error of mean and categorical data in percentages. Treatment differences between and within groups were analyzed by unpaired *t*-test or one-way analysis of variance, followed by *post hoc* Tukey's multiple comparison test for parametric data. Non-parametric data was analyzed using Mann–Whitney U-test or Kruskal–Wallis test followed by *post hoc* Dunn's multiple comparison test. Correlation between the levels of reactive oxygen species and vitiligo disease activity score was done by Spearman's correlation coefficient test.

RESULTS

The study population included 23 patients with active ($n = 17$) and stable ($n = 6$) vitiligo, of which 14 (61%) were female [Table 1]. The age and gender distribution between groups was comparable [Table 1]. The overall disease duration (years) and median (interquartile range) in active vitiligo was 6.00 (1.25–15.00), and 5.87 (0.58–39.25) in stable vitiligo [Table 1]. Vitiligo disease activity score is a six-point scale for assessing vitiligo stability wherein the scale ranges from −1 to +4. A score of −1 denotes stability for 1 year along with repigmentation, while +4 denotes highly active lesions.[18] The average vitiligo disease activity score was +3 in active vitiligo patients and was +1 in the stable variant.

Higher baseline reactive oxygen species in erythrocytes from patients with vitiligo

A higher generation of reactive oxygen species was demonstrated in erythrocytes from patients with vitiligo compared to healthy controls. The geometrical mean fluorescence channel was 23.05 ± 2.11 versus 17.77 ± 1.79, $P = 0.039$, in agreement with our previous

study.[10] Based on the disease profile, the levels of reactive oxygen species were significantly higher in patients with active vitiligo than healthy controls, the geometrical mean fluorescence channel being 24.76 ± 2.65, $P = 0.0348$ [Figure 1a and b]. The levels in patients with stable vitiligo were comparable with healthy controls, the geometrical mean fluorescence channel being 18.21 ± 2.25 [Figure 1a and c]. However, the levels of reactive oxygen species showed a trend towards a positive correlation with vitiligo disease activity score ($r = 0.37$), suggesting that they could be a feature of the disease.

Ex vivo effect of *Piper betle* on the generation of reactive oxygen species in erythrocytes

Erythrocytes are exposed to a high oxygen tension in the arterial blood and heme iron content, and are thus subjected to oxidative stress.[19] As *Piper betle* is an effective anti-oxidant, this study aimed to evaluate whether it had an impact on the generation of reactive oxygen species in erythrocytes from vitiligo patients.[12-16] It decreased the intracellular reactive oxygen species in healthy controls [Figure 2a and Table 2] and patients with active vitiligo [Figure 2b and Table 2]. In healthy controls, *Piper betle* in concentrations of 0.5 and 1.0 μg/ml reduced the baseline reactive oxygen species generation by 31.7%. This was evident by a decrease in the geometrical mean fluorescence channel from 17.77 ± 1.79 to 12.14 ± 1.03 and 12.13 ± 1.12, $P = 0.014$, respectively [Table 2]. A significant decrease was demonstrated in active vitiligo, with the reduction in reactive oxygen species generation being 47.6% and 44.3% respectively. The geometrical mean fluorescence channel decreased from 24.76 ± 2.65 to 12.97 ± 1.72, $P < 0.001$ and 13.79 ± 1.52, $P < 0.01$, respectively. However, the decrease was only 32.6% and 34.0% in stable vitiligo (data not shown).

Table 1: Study population

Parameters	Healthy controls (*n*=18)*	Patients with vitiligo (*n*=23)*	
		Active (*n*=17)	Stable (*n*=6)
*Age (years)	28.00 (25.75-30.75)	38.00 (24.50-45.00)	40.50 (20.75-51.25)
Sex (male: female)	7:11	5:12	4:2
*Disease duration (years)	NA	6.00 (1.25-15.00)	5.87 (0.58-39.25)
*Percent of total body surface area involved	NA	11.00 (5.00-20.00)	10.00 (1.75-35.00)
VIDA score	NA	+3 (+2-+4)	+1 (0-+1)

*Values are in median (IQR). IQR: Interquartile range, VIDA: Vitiligo disease activity, NA: Not applicable

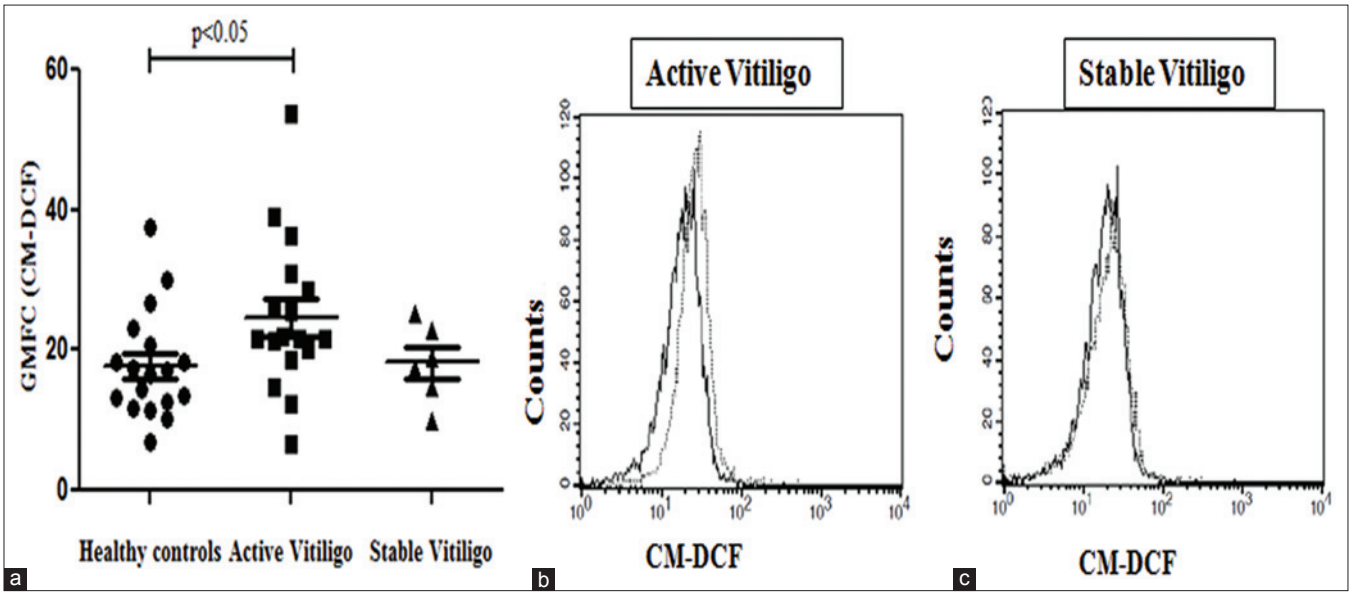

Figure 1: Flow cytometric determination of generation of reactive oxygen species in erythrocytes. (a) Scatter plot of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence in healthy controls versus patients with active or stable vitiligo. (b) Representative histogram profile of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence in a healthy control (solid line) versus active vitiligo (dotted line). (c) Representative histogram profile of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence in a healthy control (solid line) versus stable vitiligo (dotted line)

Scavenging action of *Piper betle* on hydrogen peroxide-mediated generation of reactive oxygen species in erythrocytes

Erythrocytes sourced from healthy controls and patients with vitiligo were incubated with a known pro-oxidant, hydrogen peroxide (0.5 mM), which led to an increase in the fluorescence of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein. In healthy controls, the geometrical mean fluorescence channel was increased 6.84-fold, whereas in patients with active and stable vitiligo, the increase was 7.54-fold and 9.30-fold respectively (data not shown).

Piper betle in concentrations of 0.5 and 1.0 μg/ml effectively scavenged the hydrogen peroxide in erythrocytes from healthy controls by 52.4% and 62.9% respectively [Figure 3a and Table 2]. The geometrical mean fluorescence channel decreased from 121.60 ± 18.00 to 57.87 ± 5.68, $P < 0.001$ and 45.08 ± 6.22, $P < 0.001$ in healthy controls. In active vitiligo, the enhanced fluorescence of 186.70 ± 36.10 decreased to 102.70 ± 18.96 and 80.34 ± 14.01, $P < 0.05$ with concentrations of 0.5 and 1.0 μg/ml respectively [Figure 3b and Table 2]. This resulted in a decline of fluorescence by 45.0% and 57.0% respectively. In

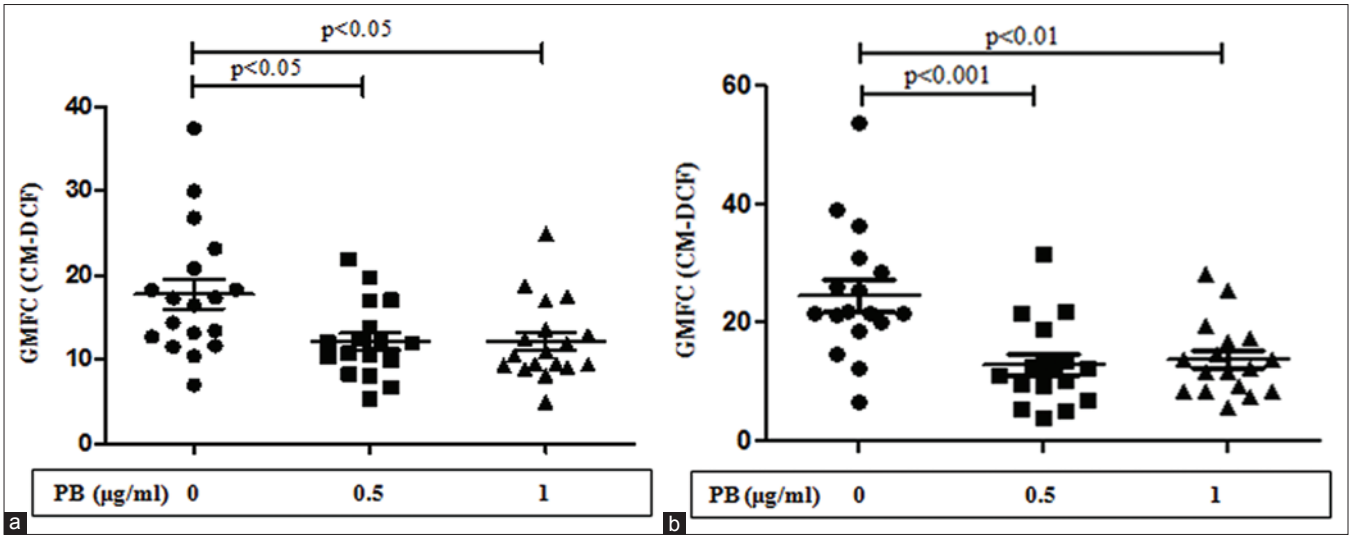

Figure 2: Effect of *Piper betle* on generation of reactive oxygen species. Erythrocytes sourced from (a) healthy controls and (b) patients with active vitiligo were incubated with *Piper betle*, labeled with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate and analyzed for fluorescence

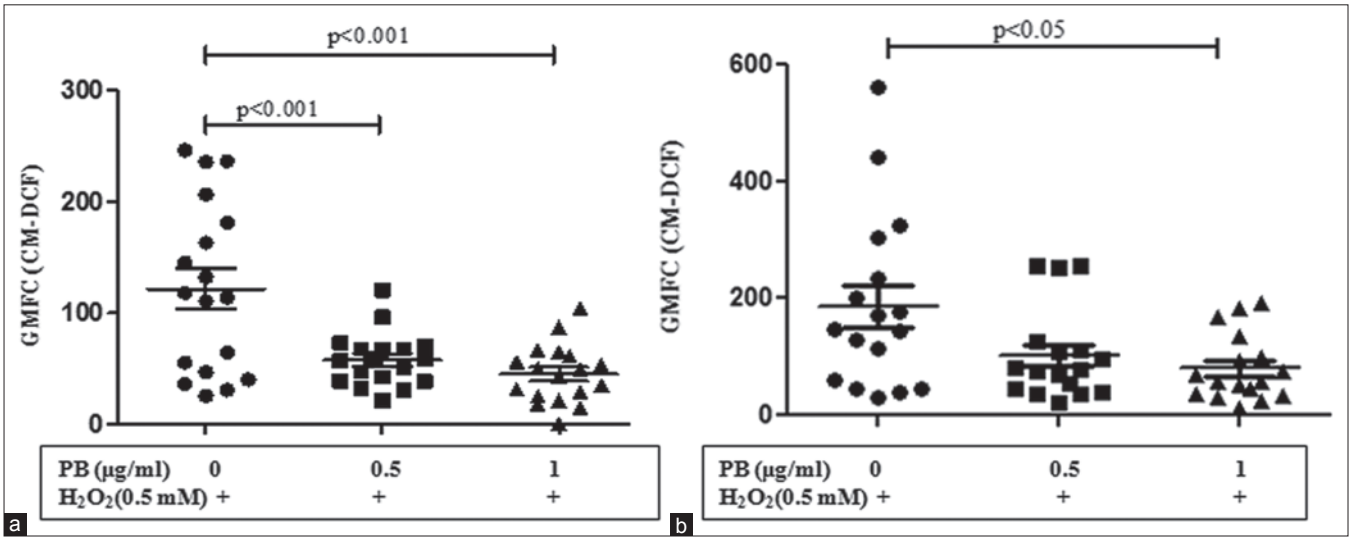

Figure 3: Scavenging action of *Piper betle* on hydrogen peroxide mediated generation of reactive oxygen species. Erythrocytes sourced from (a) healthy controls and (b) patients with active vitiligo were pretreated with *Piper betle*, followed by the addition of hydrogen peroxide. They were then labeled with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate and analyzed for fluorescence

Table 2: Effect of *Piper betle* on generation of reactive oxygen species in erythrocytes

Treatment	Healthy controls (*n*=18)	Active vitiligo (*n*=17)	*P*
-	17.77±1.79	24.76±2.65	0.034
Piper betle (0.5 μg/ml)	12.14±1.03*	12.97±1.72***	0.6748
Piper betle (1.0 μg/ml)	12.13±1.12*	13.79±1.52**	0.6089
H_2O_2 (0.5 mM)	121.60±18.00***	186.70±36.10***	0.2689
H_2O_2 + *Piper betle* (0.5 μg/ml)	57.87±5.68#	102.70±18.96	0.0622
H_2O_2 + *Piper betle* (1.0 μg/ml)	45.08±6.22#	80.34±14.01@	0.0670

*P<0.05, **P<0.01, ***P<0.001: Significantly different from baseline, #P<0.001, @P<0.05: Significantly different from treatment with H_2O_2. Erythrocytes (5×10^5) were incubated with *Piper betle* in the absence and presence of H_2O_2, and the generation of reactive oxygen species was measured in terms of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence as described in the materials and methods. All comparisons of means were done by an independent sample *t*-test. H_2O_2: Hydrogen peroxide

stable vitiligo cases, *Piper betle* in concentrations of 0.5 and 1.0 μg/ml also scavenged hydrogen peroxide. This led to a decrease in fluorescence by 55.0% and 64.0% respectively (data not shown).

DISCUSSION

Oxidative stress has been suggested to play a contributory role in the pathogenesis of vitiligo. This is supported by evidence of a redox imbalance, systemically and at the site of pathology.[5,20-22] Levels of lipid peroxidation were raised in patients with vitiligo, more so in those with active disease.[9] The enhanced generation of reactive oxygen species in erythrocytes from patients with active vitiligo, as compared to stable vitiligo and healthy controls [Figure 1] was in concordance with our previous study.[10] Furthermore, the levels of reactive oxygen species showed a trend toward positive correlation with vitiligo disease activity score, implying that it may be a feature of the disease.

Characterization of pro-inflammatory based signaling pathways in melanocyte cultures acquired from vitiligo non-lesional skin has shown hyperactivation of mitogen-activated protein kinase and cyclic adenosine monophosphate response element binding proteins, resulting in their enhanced phosphorylation.[23] Components of the nuclear factor E2-related factor 2/antioxidant response element pathway and its downstream antioxidant enzyme heme oxygenase-1, are pivotal for melanocytes to handle hydrogen peroxide induced oxidative stress.[24] As melanocytes sourced from patients with vitiligo showed reduced nuclear translocation of nuclear factor E2-related factor 2 and decreased expression of heme oxygenase-1, the impairment of this pathway could potentially generate a redox imbalance leading to increased melanocyte destruction.[25]

Plant-derived compounds with anti-oxidant properties are emerging as an attractive option for the treatment of vitiligo as an adjunct to phototherapy.[26] Oral supplementation with an anti-oxidant pool (consisting of alpha-lipoic acid, vitamin C, vitamin E and polyunsaturated fatty acids), before and during narrow band ultraviolet B therapy increased the efficacy of treatment.[26] A combination of *Phyllanthus emblica* fruit extract, vitamin E and carotenoids used concomitantly with topical therapy and phototherapy resulted in mild repigmentation and a steady state in terms of disease activity.[27] Experimental models of vitiligo have yielded similar results, in which mice treated with monobenzone developed depigmentation, whereas treatment with epigallocatechin-3-gallate resulted in a delay of depigmentation and reduction in the depigmented area.[28] Epigallocatechin-3-gallate also prevented the increase of perilesional $CD8^+$ T cells and levels of circulatory pro-inflammatory cytokines such as tumor necrosis factor-α, interferon-γ and interleukin-6.[28] Conversely, studies have indicated no improvement with the inclusion of anti-oxidant therapy when prescribed along with photochemotherapy.[29] The topical application of anti-oxidant gels also failed to improve the lesions when treated along with narrow band-ultraviolet B.[30] Considering all these points, it is essential to undertake carefully designed clinical trials with large sample sizes to understand the role of anti-oxidants in vitiligo. The *ex vivo* treatment of erythrocytes with *Piper betle* significantly decreased the generation of reactive oxygen species in patients with active vitiligo [Figure 2 and Table 2]. Furthermore, as it also decreased the generation of reactive oxygen species in erythrocytes treated with hydrogen peroxide, [Figure 3 and Table 2], it endorses the ability of this plant extract to scavenge reactive oxygen species effectively.

The present study is limited because of its small sample size. The anti-oxidant activity of *Piper betle* has been established in reducing the generation of reactive oxygen species in erythrocytes. This could then be further investigated to establish its role on melanocytes obtained from vitiligo patients, to assess its effects on the skin.

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Conflicts of interest

There are no conflicts of interest.

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