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Antioxidant effect of ethanolic extract of *Piper betle* Linn. (Paan) on erythrocytes from patients with HbE-beta thalassemia

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HbE-beta thalassemia is caused by an interaction between HbE and defective β globin gene of thalassemia. Repeated blood transfusions cause an iron overload, triggering an enhanced generation of free radicals. In the present study, the anti-oxidant property of ethanolic extract of the leaves of *Piper betle* Linn. (PB) was evaluated in the erythrocytes from patients with HbE-beta thalassemia. In patients with HbE-beta thalassemia (n = 30) and age- and sex-matched healthy individuals (n = 30), the baseline level of reactive oxygen species (ROS) and free radical scavenging activity in the erythrocytes was measured by flow cytometry using dihydrodichlorofluorescein diacetate (H_2DCFDA), in terms of the geometric mean fluorescence channel (GMFC). The baseline generation of ROS was significantly higher in the erythrocytes from patients with HbE-beta thalassemia, as compared to healthy volunteers, the GMFC being 67.20 ± 4.64 vs. 23.03 ± 1.88 ($p<0.001$), which was effectively decreased by PB. Similarly, H_2O_2 (0.5-1.0 mM) induced a higher increase in the GMFC in the erythrocytes from patients with HbE-beta thalassemia, as compared to controls which was effectively reduced by PB. Taken together, PB showed promising anti-oxidant activity against the erythrocytes from patients with HbE-beta thalassemia.

Keywords: HbE beta thalassemia, Reactive oxygen species (ROS), *Piper betle* Linn., Anti-oxidant, Erythrocytes

Thalassemia, a common genetic disorder is characterized by impaired biosynthesis of the globin chain, resulting in a variable degree of microcytic hypochromic anemia, due to a combined effect of ineffective erythropoiesis and premature hemolysis[1]. In South-East Asia, a commonly occurring hemoglobinopathy is HbE-beta thalassemia caused by an interaction between HbE, an abnormal Hb variant and the defective β globin gene of thalassemia[2]. It has been estimated that frequency of the HbE disease carrier state varies from 5-50% in India and most of them are reported from Eastern and North-Eastern India. Recently, an increased number of reports have been described mainly amongst Bengalis in Eastern India[2]. The disease is characterized by a wide range of clinical severity which ranges from mild to moderately severe transfusion dependent anemia, often indistinguishable from beta thalassemia major.

Research in the field of molecular pathology of thalassemia has revealed that damage to various cellular components, particularly the erythrocyte membrane is associated with increased oxidative damage[3]. Iron overload has been implicated as the main precipitating factor for generation of excess free radicals. Iron, being a potent reagent in Fenton reaction catalyzes breakdown of hydrogen peroxide (H_2O_2), resulting in formation of highly toxic hydroxyl ($OH^{\cdot}$) radicals[4]. The reactive $OH^{\cdot}$ radicals can eventually attack and oxidize various cellular macromolecules, mainly membrane lipids and proteins[5]. In beta thalassemia, reduced levels of non-enzymatic antioxidants like reduced glutathione (GSH)[3], glutathione peroxidase and glutathione reductase[6] along with vitamins A, E, as also enzymatic antioxidants such as superoxide dismutase[7] and catalase[8] have been reported. Studies have shown that even mild oxidative stress makes HbE more unstable, resulting in precipitation and worsening of anemia,

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Abbreviations: FSC, forward scatter; GMFC, geometric mean fluorescence channel; H_2DCFDA, 2',7'-dichlorofluorescin diacetate; PB, ethanolic extract of *Piper betle* leaves; PE, phycoerythrin; ROS, reactive oxygen species; SSC, side scatter.

particularly during febrile episodes[9].

Considering the role of oxidative stress in the pathophysiology of thalassemia, treatment with anti-oxidants is an attractive option. Various compounds with potential anti-oxidant activity have been tested both *in vivo* and *in vitro* like α-tocopherol, N-acetyl cysteine, coenzyme Q10 and hydroxyurea[10-12]. Efforts are also being focused on testing plant-derived biomolecules, as they have the added advantage that they can be used for prolonged periods with minimal adverse effects.

Among the vast treasury of Indian medicinal plants, *Piper betle* (Family: Piperaceae) leaves have long been studied for their diverse pharmacological actions[13] such as anti-inflammatory[14,15], anti-oxidant[16], radioprotective[17] and anti-allergic activities[18]. They have also shown antimicrobial[19], antifungal[20], anti amoebic[21], antifertility[22] and antiplatelet activities[23].

In the present study, the anti-oxidant potential of ethanolic extract of leaves of *P. betle* Linn. has been evaluated against the enhanced oxidative stress generated in the erythrocytes of patients with HbE-beta thalassemia.

Materials and Methods

Chemicals

All chemicals used were of analytical grade and obtained from Sigma Aldrich (St Louis, MO, USA), except for anti-CD235a antibody (BD Biosciences, San Jose, CA, USA).

Preparation of ethanolic extract

Fresh leaves of *Piper betle* Linn. were obtained locally and authenticated by the Botanical Survey of India, Shibpur, Howrah. Leaves were air-dried, chopped, powdered in a hand crusher and processed as previously described[14]. The resultant extract referred to as PB was dissolved in propylene glycol and stored at 4°C (10 mg/ml).

Study population

The study population included healthy individuals (n=30) and patients with HbE beta thalassemia (n=30). Patients were recruited from the Thalassemia Clinic, Institute of Haematology and Transfusion Medicine, Kolkata. As per the study protocol, all patients who had been polytransfused (>20 blood transfusions) and had received regular blood transfusions (2-3 units of packed cells every 4-6 weeks), depending on severity of anemia were included. Subjects with serum ferritin levels <1000 ng/ml and who had received blood transfusions within 1 month prior to entry into the study were excluded. Also excluded were subjects with other concurrent medical illnesses e.g. cardiovascular diseases, hepatic disorders, other transfusion-related diseases, any acute illnesses as also pregnant/lactating females. Informed consent was obtained from all subjects and in case of minors, parental written consent was taken and the study protocol received prior approval from the Institutional Ethics Committee.

Labeling of erythrocytes with surface marker (anti-CD235a)

Erythrocytes were gated using a phycoerythrin (PE)-labeled anti-CD235a, an antibody specific for glycophorin A (CD235a) that is selectively expressed on the erythrocytic surface[24]. They were stained with either PE conjugated isotype or anti-CD235a and incubated for 15 min in dark, followed by washing and resuspension in sheath fluid. Finally, cells were acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) on bivariate dot plots using Forward scatter (FSC) vs. Side scatter (SSC) to gate the cell population and SSC vs. FL2 to quantify labeling of anti-CD235a.

Measurement of reactive oxygen species (ROS) in erythrocytes

Dihydrodichlorofluorescin diacetate (H_2DCFDA) is a non-fluorescent dye that freely enters the cell; after hydrolytic cleavage by intracellular esterases, the non-fluorescent dihydrodichlorofluorescein (H_2DCF) is produced which is impermeable and hence retained by the cell. H_2DCF is then oxidized by intracellular oxidants to the highly fluorescent dichlorofluorescein (DCF) which can be measured on a flow cytometer as the geometric mean fluorescence channel (GMFC). Thus, measurement of fluorescence of DCF is a good indicator of the intracellular ROS generated[25]. For optimization of effective concentration of H_2DCFDA in erythrocytes, erythrocytes (1×10^6/ml) were incubated for 30 min at 37°C with H_2DCFDA (10-400 μM). A total of 10,000 cells were acquired on a flow cytometer using a bivariate FSC vs. SSC plot to gate the population and a FL1 histogram to quantify the fluorescence. Finally, analysis was done using BD cell Quest Pro Software to detect GMFC.

Determination of non-toxic concentrations of PB

For determining non-toxic concentrations of PB, cells (1×10^6/ml) were incubated with PB (0.1-10.0 μg/ml) for 1 h at 37°C and subsequently incubated with H_2DCFDA (50 μM) at 37°C for 1 h. Cellular morphology was analyzed by flow cytometry as

described above.

Determination of ROS generation in erythrocytes

To measure baseline intracellular ROS, erythrocytes (1×10^6/ml) from normal donors and patients with HbE-beta thalassemia were incubated with H_2DCFDA (50 μM) for 30 min at 37°C and analyzed by flow cytometry.

Evaluation of PB on generation of ROS in erythrocytes

To study the effect of PB on baseline generation of ROS, both normal and thalassemic erythrocytes were initially treated with PB (0.5-1.0 μg/ml) for 1 h at 37°C, followed by labeling with H_2DCFDA (50 μM) and processed as described above.

Effect of PB on scavenging of ROS in erythrocytes

ROS was generated by the addition of H_2O_2. To identify an effective concentration of H_2O_2 to induce oxidative stress in erythrocytes without hampering cell viability, H_2O_2 (0.25-2.0 mM) was added for 1 h at 37°C. Cells were then centrifuged (3000 rpm × 5 min), resuspended in sheath fluid containing H_2DCFDA (50 μM) and morphology was analyzed by flow cytometry. It was observed that cell viability was unaffected up to 1 mM H_2O_2 and accordingly, all subsequent experiments were performed using 0.5-1.0 mM H_2O_2.

To study the scavenging action of PB, erythrocytes were pre-incubated with PB (0.5-1.0 μg/ml), followed by H_2O_2 (0.5 or 1 mM) for 1 h at 37°C and then processed for acquisition as described above.

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by STATISTICA version 6. For between group variables, unpaired '*t*' test and for within group analysis of the effect of PB, paired '*t*' test was used and differences were considered significant when $p<0.05$.

Results and Discussion

Study population

Patients with HbE-beta thalassemia were aged between 8-44 yrs, had a sex ratio of 19:11 (male: female) along with age (7-45 yrs) and sex-matched (21:9) healthy individuals (Table 1). Depending on their serum ferritin levels, a majority of patients had received iron chelation as desferoxamine (20-40 mg/kg s.c. infusion) or deferiprone (75 mg/kg oral); importantly, none had received any antioxidant therapy.

Flow cytometric analysis of anti-CD235a labeling

Erythrocytes were gated by labeling with anti-CD235a, an established specific erythrocyte marker[24] using a 2 parameter dot plot and fluorescence was specified using a FL2 quadrant plot (Fig. 1). As

Table 1—Study population

Parameters	Healthy donors (n = 30)	HbE beta thalassemia (n = 30)
Age range (Median ± IQR yrs)	7-45 (23.50 ± 17.00)	8-44 (20.00 ± 10.00)
Sex	Male = 21 Female = 9	Male = 19 Female = 11
M : F	2.3 : 1	1.8 : 1
Duration of illness range (Median ± IQR yrs)	NA	4-40 (15.00 ± 9.50)
Units of blood received Range (mean ± SD)	NA	20-170 (78.17 ± 33.59)
Ferritin level (mean ± SD, ng/ml)	NA	1115–3000 (1602.32 ± 584.06)
Splenectomy	NA	Yes: 16 (53.33%) No: 14 (46.67%)
Fe chelation received	NA	Yes: 10, No: 10, Irregular : 6
Antioxidants received	No	No

NA, Not applicable

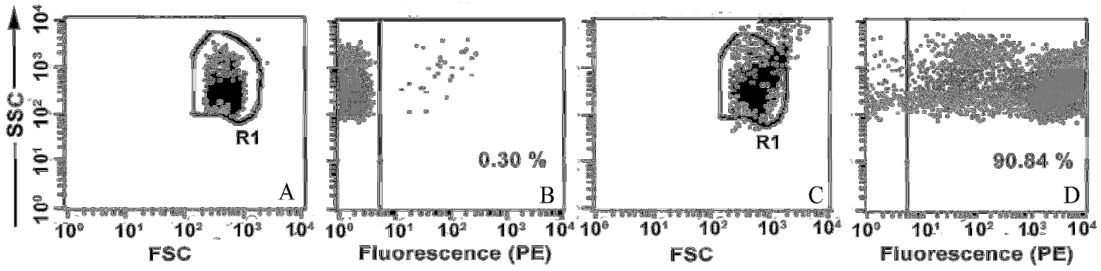

Fig. 1—Immunophenotyping of erythrocytes [Representative profile of a bivariate dot plot (A, C) and quadrant plot (B, D) showing binding of anti-CD235a (PE) in erythrocytes (D) as compared to isotype control (B). Erythrocytes were stained with PE labeled anti-CD235a and processed as described in 'Materials and Methods']

fluorescence was detected in almost all identified cells, positivity being 90.84% (D) in anti-CD235a labeled cells (C), as compared to isotype-matched cells (A) with only 0.30% positivity (B), the cells were confirmed to be erythrocytes.

Assay standardization for measurement of oxidative stress

To identify the optimum concentration of H_2DCFDA needed for measurement of generation of ROS by flow cytometry, the erythrocytes were initially labeled with H_2DCFDA (10-400 μM) and analyzed on a flow cytometer. Increasing concentrations of H_2DCFDA (10-50 μM) increased the GMFC in a dose-dependent manner from 9.49 ± 1.00 (10 μM), 11.79 ± 1.34 (25 μM) to 16.74 ± 2.03 (50 μM). However, increasing the concentration further (400 μM) did not proportionately increase the GMFC (15.35 ± 0.49). The data indicated that saturation was attained at 50 μM H_2DCFDA and was accordingly selected as the appropriate concentration for study of the erythrocytes.

To optimize concentration of H_2O_2 that would be an effective pro-oxidant without hampering cell viability, the erythrocytes were incubated with H_2O_2 (0.25-2.0 mM) and analyzed by flow cytometry. With increasing concentrations of H_2O_2 up to 1.0 mM, the GMFC progressively increased from 30.51 ± 3.58 (0.25 mM), 47.28 ± 4.25 (0.5 mM) to 64.14 ± 6.48 (1.0 mM), while background fluorescence was 12.00 ± 1.05. However, with a further increase in the concentration of H_2O_2 to 1.5 mM, the GMFC decreased to 36.07 and with 2 mM H_2O_2 decreased even further to 19.08. This was attributed to cell death induced by H_2O_2, accordingly, two doses of H_2O_2, 0.5 and 1.0 mM were selected.

As PB was dissolved in propylene glycol (0.5% v/v, PG), it was important to confirm that the vehicle had no effect on generation of ROS. Accordingly, the erythrocytes were incubated with 0.5% PG representing the highest concentration used. In the erythrocytes incubated with PG, the GMFC was similar to untreated erythrocytes (being 18.78 ± 0.34 vs. 19.47 ± 0.01, respectively), indicating that PG (0.5% v/v) had no effect on generation of ROS and could be used as a vehicle in the study.

Determination of non-toxic concentrations of PB on erythrocytes

As toxic concentrations of PB could themselves decrease the GMFC by hampering cell viability, it would be difficult to interpret whether the decrease in the GMFC was due to an antioxidant action of PB or simply its toxic action. Accordingly, non-toxic concentrations of PB were determined. As the erythrocytes are non-nucleated structures, no biochemical assay could be performed to evaluate cell viability; instead, we selected cellular morphology as the criteria for cell viability by measuring forward and side scatter characteristics (FSC and SSC) of the erythrocytes.

With PB up to 1.0 μg/ml, there was no change in either morphology or GMFC. However, higher concentrations of PB (2.5-10.0 μg/ml) caused morphological changes and accordingly, two concentrations of PB i.e. 0.5 and 1.0 μg/ml were chosen for all subsequent experiments.

Higher baseline ROS was generated in erythrocytes from patients with HbE-beta thalassemia

The baseline ROS generated in normal and thalassemic erythrocytes was measured, following addition of H_2DCFDA (50 μM) and a representative profile is shown in Fig. 2. In the erythrocytes from patients with HbE-beta thalassemia, the basal level of ROS was 2.9-fold higher than erythrocytes from healthy donors being 67.20 ± 4.64 vs. 23.03 ± 1.88, $p<0.001$. The higher baseline ROS confirmed that patients with HbE-beta thalassemia also suffered from increased oxidative stress like beta thalassemia[3].

As red cells mature, they lose their nucleus, ribosomes and mitochondria resulting in an absence of further protein synthesis. However, due to the high O_2 tension in arterial blood and heme Fe^{2+} content, the generation of ROS in red cells is an ongoing

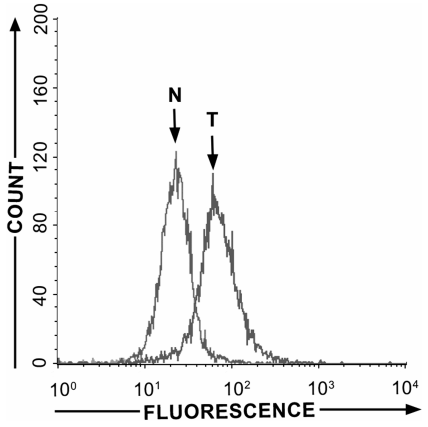

Fig. 2—Baseline ROS generated in erythrocytes from normal donors and patients with HbE-beta thalassemia [A representative profile of erythrocytes from healthy donors (N) and patients with HbE thalassemia (T) that were labeled with H_2DCFDA (50 μM) and assayed flow cytometrically as described in 'Materials and Methods']

Table 2—Effect of PB on scavenging of H_2O_2 in erythrocytes

[Erythrocytes from healthy controls or patients with HbE-beta thalassemia were analyzed for scavenging activity as described in'Materials and Methods']

H_2O_2 (mM)	PB (µg/ml)	Healthy controls (n = 30) % decrease in GMFC[#]	Thalassemia (n = 30) % decrease in GMFC[#]
0.5	0.5	47.27	37.92
	1.0	48.39	37.90
1.0	0.5	45.38	39.42
	1.0	47.46	40.82

[#]The % decrease in GMFC was calculated by:
$\frac{H_2O_2 \text{ mediated GMFC} - \text{GMFC after treatment with PB} \times 100}{H_2O_2 \text{ mediated GMFC}}$

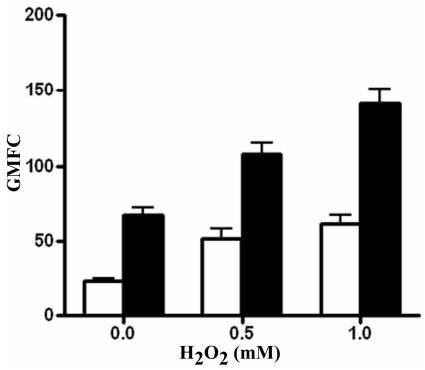

Fig. 3—Pro-oxidant effect of H_2O_2 on erythrocytes from normal donors and patients with HbE-beta thalassemia [Erythrocytes from healthy donors (n =30, □) and patients with HbE-beta thalassemia (n = 30, ■) were treated with H_2O_2 (0.5-1.0 mM) and subsequently processed for flow cytometric analysis as described in 'Materials and Methods'. Results represent mean ± SEM of the GMFC of at least three experiments in duplicate]

process. Furthermore, the erythrocytes being the major oxygen transporters have to withstand a substantial amount of oxidative stress during their entire life span, owing to the generation of oxidizing radicals such as O_2^-, H_2O_2 and HO in the erythrocytes. The erythrocytes are, therefore, endowed with potent antioxidant protection consisting of enzymatic (superoxide dismutase and catalase) as also non-enzymatic pathways (glutathione) that are capable of modifying ROS into relatively less reactive intermediates[26].

PB decreased baseline generation of ROS in both normal and thalassemic erythrocytes

To assess the antioxidant potential of PB on the erythrocytes, both normal and thalassemic erythrocytes were initially treated with PB (0.5 or 1.0 µg/ml). It was observed that PB (0.5 and 1.0 µg/ml) caused a dose-dependent reduction in GMFC in the erythrocytes from healthy donors (42.21% and 47.11% respectively, $p<0.001$) and patients with HbE-thalassemia (37.99% and 44.70% respectively, $p<0.001$).

PB showed increased scavenging activity in both normal and thalassemic erythrocytes

To enhance baseline ROS, the erythrocytes were incubated with H_2O_2 (0.5-1.0 mM). It was observed that in both groups, exogenously administered H_2O_2 (0.5-1.0 mM) induced a dose-dependent oxidative stress, the increase in GMFC being significantly higher in thalassemic erythrocytes as compared to controls following the addition of both 0.5 mM H_2O_2 (108.11 ± 7.48 vs. 51.80 ± 6.49, $p<0.001$) and 1.0 mM H_2O_2 (141.62 ± 9.59 vs. 61.04 ± 6.22, $p<0.001$, Fig. 3).

The scavenging action of PB was effectively demonstrated in the erythrocytes preincubated with PB followed by H_2O_2. PB (0.5 and 1.0 µg/ml) effectively curtailed the GMFC increase triggered by H_2O_2 (0.5 mM) in the erythrocytes from both normal donors (47.27% and 48.39%, $p<0.001$, Table 2) and patients with HbE-beta thalassemia (37.92% and 37.90%, $p<0.001$, Table 2). Similarly, in the presence of an even higher concentration of H_2O_2 (1.0 mM), both doses of PB (0.5 and 1.0 µg/ml) decreased the GMFC in both normal (45.38% and 47.46%, $p<0.001$, Table 2) and thalassemic (39.42% and 40.82%, $p<0.001$, Table 2) erythrocytes. Taken together, it was concluded that PB at both doses (0.5 and 1.0 µg/ml) effectively scavenged H_2O_2 (0.5-1.0 mM), resulting in a dose-dependent decrease in the GMFC in both normal and thalassemic erythrocytes (Table 2), corroborating a previous study that had shown PB to exert its anti-oxidant activity by increasing the activity of free radical scavengers[16].

Conclusion

The study has established that the erythrocytes from patients with HbE-beta thalassemia have higher baseline levels of ROS than healthy individuals. It has

also demonstrated the anti-oxidant potential of the ethanolic extract of leaves of *P. betle* Linn. The oxidant-scavenging efficacy of *P. betle* in the erythrocytes from patients with HbE-beta thalassemia suggests that it could attenuate the effects of iron overload in thalassemia. The major constituents of *P. betle* include phenols, chevibetol, allylpyrocatechol and their respective glycosides[15,27], however, it is important to identify the active principle(s) responsible for its antioxidant activity.

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References

1 Edward J & Benz J R (2001) In: *Harrison's Principles of Internal Medicine*, 15th edn, pp. 666-673, McGraw-Hill Companies, USA
2 Krishnamurti L (2000) *J Pediatr Hematol Oncol* 22, 558-563
3 Amer J, Goldfarb A & Fibach E (2004) *Cytometry A* 60, 73-80
4 Murray R K (2000) In: *Harper's Biochemistry* (Murray R K, Granner D K, Mayes P A & Rodwell V W, eds), 25th edn, pp.763-767, McGraw Hill Companies, USA
5 Halliwell B & Gutteridge J M C (1999) *Free Radicals in Biology and Medicine*, 3rd edn, pp. 617-624, Oxford University Press, New York
6 Kassab-Chekir A, Laradi S, Ferchichi S, Haj Khelil A, Feki M, Amri F, Selmi H, Bejaoui M & Miled A (2003) *Clin Chim Acta* 338, 79-86
7 Dhawan V, Kumar Kh R, Marwaha R K & Ganguly N K (2005) *Indian Pediatr* 42, 1141-1145
8 Scott M D (2006) *Free Radic Biol Med* 40, 1264-1272
9 Rees D C, Clegg J B & Weatherall D J (1998) *Blood* 92, 2141-2146
10 Rund D & Rachmilewitz E (2005) *N Engl J Med* 353, 1135-1146
11 Kalpravidh R W, Wichit A, Siritanaratkul N & Fucharoen S (2005) *Biofactors* 25, 225-234
12 Ajanta H, Chakraborty S, Madhusnata D, Bhattacharya D K & Manisha D (2002) *J Assoc Physicians India* 50, 1034-1035
13 Nadkarni K M (1976) In: *Indian Materia Medica*, 3rd edn, pp. 960-964, Popular Prakashan, Bombay
14 Ganguly S, Mula S, Chattopadhyay S & Chatterjee M (2007) *J Pharm Pharmacol* 59, 711-718
15 Sarkar D, Saha P, Gamre S, Bhattacharjee S, Hariharan C, Ganguly S, Sen R, Mandal G, Chattopadhyay S, Majumdar S & Chatterjee M (2008) *Int Immunopharmacol* 8, 1264-1271
16 Choudhury D & Kale R K (2002) *Phytother Res* 16, 461-466
17 Bhattacharya S, Subhramanian M, Roychoudhury S, Bauri A K, Kamat J P, Chattopadhyay S & Bandyopadhyay S K (2005) *J Radiat Res* (Tokyo) 6, 165-171
18 Wirotesangthong M, Inagaki N, Tanaka H, Thanakijcharoenpath W & Nagai H (2008) *Int Immunopharmacol* 8, 453-457
19 Shitut S, Pandit V & Mehta B K (1999) *Cent Eur J Public Hlth* 7, 137-139
20 Trakranrungsie N, Chatchawanchonteera A & Khunkitti W (2008) *Res Vet Sci* 84, 80-84
21 Sawangjaroen N, Phongpaichit S, Subhadhirasakul S, Visutthi M, Srisuwan N & Thammapalerd N (2006) *Parasitol Res* 98, 588-592
22 Sarkar M, Gangopadhyay P, Basak B, Chakraborty K, Banerji J, Adhikary P, Chatterjee A (2000) *Contraception* 62, 271-274
23 Lei D, Chan C P, Wang Y J, Wang T M, Lin B R, Huang C H, Lee J J, Chen H M, Jeng J H & Chang M C (2003) *J Agric Food Chem* 51, 2083-2088
24 Tarpey M M, Wink D A & Grisham M B (2004) *Am J Physiol Regul Integr Comp Physiol* 286, R431-R444
25 Cimen M Y (2008) *Clin Chim Acta* 390, 1-11
26 Rathee J S, Patro B S, Mula S, Gamre S, Chattopadhyay S (2006) *J Agric Food Chem* 54, 9046-9054