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Effects of leaf extracts of selected medicinal plants on blood against hypotonic solution by measuring optical density: An *in vitro* study

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The medicinal plant leaf extracts alter the morphology of the cellular components of blood, especially RBCs. However, information on biochemical changes occurring during these events *in vitro* is lacking. Here, we studied the effect of medicinal plant [*Azadirachta indica* A. Juss (Neem), *Emblica officinalis* Gaertn. (Indian Gooseberry or Amla) and *Ocimum tenuiflorum* L. (Holi basil or Tulsi)] leaf extracts as against the hypotonic solution by measuring the optical density of blood at various wavelengths at 0 and 30 min. The blood, exposed to hypotonic solution, showed cellular degradation and higher protein metabolism; higher glucose metabolism and oxidative cellular damage as well as higher oxygen absorption as compared to test samples. Among the three leaf extracts studied, the Amla extract treatment had comparatively more protective role for cellular components of the blood as against hypotonic solution. However, Tulsi extract significantly reduced hemolysis, heme catabolism and redox stress in RBCs. Tulsi also increased glucose metabolism indicating its antidiabetic property. To conclude, Amla leaf extract significantly reversed the effects of the hypotonic solution on the cellular components while Tulsi extract exhibited therapeutic role in maintaining homeostasis of cellular components in blood.

Keywords: Antidiabetic, Amla, *Azadirachta indica*, CO-Hb, Hemolysis, Herbal, Holi basil, Homeostasis, Indian gooseberry, Neem, *Ocimum tenuiflorum*, oxy-Hb, Redox stress, Tulsi

Extracts of medicinal plants have been used in preparation of traditional medicines in several countries *viz*. India[1-5] China[6], Iran[7], Africa[8-11], Pakistan[12], Turkey[13], etc. The plant extracts may act as antiarthritic[1,14], anticancer[1,3], antidepressant[3], antipyretic[1,3], antioxidant[3,4,14], antidiabetic[1,15], immunostimulatory[4] antifatigue[1,4], anti-microbial[1,3,16], anti-inflammatory[1,3,7,14,17], antiviral[1,5], antiplasmodial[9], radio-[18] broncho-[3], renal-[13] and hepato-protective[2,3,13] agents or to treat gut ailments[19], skin ulcer[10], blood stasis syndrome[6], etc. Jamshidi & Cohen[20] have reviewed the clinical efficacy of Tulsi (Holy basil) particularly for its antimicrobial, anti-inflammatory, adaptogenic, cardioprotective, and immuno-modulatory effects. Recently, Sarkar *et al*.[21] have isolated a novel compound from neem with antileukemic potential.

Though these extracts in optimum quantities exhibit healing properties, higher dosages may have deleterious effects[22,23] which may be manifested as morphological and/or biochemical changes in red blood corpuscles (RBCs) and other cells in the blood. Moreover, blood is the carrier of all the metabolites, toxic substances, gases, etc. from one tissue to the other. The extracts, when consumed in the form of medicine, first enter the circulation where they might influence biochemical parameters of the blood.

Of all the cellular components of blood, the RBCs are most prevalent that carry out the most important function of the body i.e. exchange of gases between the cells and blood. Various factors may affect the function of the RBCs like the quality of air, the structural properties of RBCs, the biochemical environment of the blood, other external factors, etc. Supplementation of green tea is known to ameliorate oxidative stress induced damage on RBC morphology and membrane properties[24]. Hypotonic solution and leaf extracts of *Azadirachta indica* A. Juss (Neem), *Phyllanthus emblica* L. (Amla or Indian Gooseberry) and *Ocimum tenuiflorum* L. (Tulsi or Holi basil)have been reported to influence the morphology of the RBCs *in vitro*[25,26]. Here, we studied the changes in various biochemical parameters of blood at a suitable concentration of extracts of Tulsi, Neem and Amla as used in our earlier study on morphological parameters[25] and to ascertain any therapeutic or protective role of these extracts.

Materials and Methods

Selection of plants and preparation of leaf extracts

The leaves of selected medicinal plants (Neem, Amla and Tulsi) were collected from the college premises and the surrounding areas during Jan-April, 2017. The plants were identified and authenticated by Dr Rama Sisodia, Department of Botany, Maitreyi College, Delhi. The leaves were washed thoroughly

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with distilled water, dried and powdered. The powder was used to prepare a working concentration of 0.3 mg/mL with tris-HCl (pH 6.8) and the solution was filtered through fine loin cloth to get the working leaf extract. The preparation of leaf extract was adapted from the method as described by Mitra *et al*.[27].

Collection of blood and experimental set-up

The chicken (*Gallus gallus domesticus*) blood was collected from the butcher house early morning on the day of the experiments in tubes rinsed with ethylene diamine tetra acetic acid (EDTA), the anticoagulant, and brought to the laboratory. The blood was diluted to 1:100 with phosphate buffered saline (PBS). The diluted blood was mixed with equal volumes of various test substances as per the following design[28] and incubated at room temperature ranging from 15-28°C. Group I: Blood + hypotonic solution (0.4% NaCl solution); Group II-IV: Blood + hypotonic solution + Neem Amla & Tulsi leaf extract (50:50), respectively. Each of the experiments in four groups was kept in two sets: one, the optical density (OD) to be measured at 0 min i.e. immediately after mixing, and second, OD to be measured after 30 min of incubation. The samples were centrifuged at 10000 rpm for 10 min in REMI centrifuge. The supernatant was isolated and the optical density was measured using Systronics UV-VIS Spectrophotometer Type 118 at 215 nm (protein), 280 nm (amino acids), 346 nm (NADH and NADPH), 418 nm, 544 nm & 578 nm [carboxy-hemoglobin: (CO-Hb) and oxy-hemoglobin (oxy-Hb)][29].

Statistical analysis

The data was analyzed by paired sample t-test, using SPSS version 20.0, IBM Corp., USA, to compare the means of the ODs of the samples at 0 and 30 minutes after incubation. The results are presented as mean±SD and the differences were considered statistically significant at $P < 0.05$.

Results and Discussion

The changes in the optical densities were recorded and tabulated as given in Table 1 and Fig. 1. The OD of blood at 215 nm corresponds to the concentration of protein in the sample. In control, there were higher OD

Table 1: Optical densities of blood at various wavelengths after various treatments

Wavelength	OD at 0 minutes (Mean ± SD)	OD after 30 minutes (Mean ± SD)	Result of paired sample 't' test
Treatment of blood with hypotonic solution			
215 nm	0.324±0.037	0.273±0.005* ↓	t (3) = 8.340; $P = 0.004$
280 nm	0.304±0.004	0.517±0.004** ↑	t (3) = –147.224; $P < 0.001$
346 nm	1.032±0.04	0.649±0.006** ↓	t (3) = 17.009; $P < 0.001$
418 nm	1.309±0.006	0.594±0.004** ↓	t (3) = 240.571; $P < 0.001$
544 nm	0.862±0.006	0.551±0.003** ↓	t (3) = 105.178; $P < 0.001$
578 nm	0.097±0.004	0.533±0.004** ↑	t (3) = –168.009; $P < 0.001$
Treatment of blood with Tulsi leaf extract and hypotonic solution			
215 nm	0.031±0.003	0.053±0.002* ↑	t (3) = –12.429; $P = 0.001$
280 nm	0.879±0.009	0.917±0.003* ↑	t (3) = –7.825; $P = 0.004$
346 nm	0.493±0.002	0.685±0.003** ↑	t (3) = –95.383; $P < 0.001$
418 nm	1.617±0.004	1.022±0.027** ↓	t (3) = 39.159; $P < 0.001$
544 nm	1.935±0.005	0.229±0.002** ↓	t (3) = 609.545; $P < 0.001$
578 nm	1.686±0.003	0.233±0.003** ↓	t (3) = 528.273; $P < 0.001$
Treatment of blood with Amla leaf extract and hypotonic solution			
215 nm	0.041±0.004	0.041±0.002	t (3) = 0.162; $P = 0.882$
280 nm	0.254±0.002	0.233±0.003* ↓	t (3) = 15.399; $P = 0.001$
346 nm	0.124±0.003	0.122±0.002	t (3) = 0.926; $P = 0.423$
418 nm	0.971±0.048	0.995±0.004 ↑	t (3) = –0.971; $P = 0.403$
544 nm	1.435 ±0.187	1.296±0.002 ↓	t (3) = 1.481; $P = 0.235$
578 nm	1.41±0.268	1.058±0.003 ↓	t (3) = 2.605; $P = 0.08$
Treatment of blood with Neem leaf extract and hypotonic solution			
215 nm	0.044±0.001	0.052± 0.002* ↑	t (3) = –13.113; $P = 0.001$
280 nm	0.881±0.001	0.902±0.001** ↑	t (3) = –19.696; $P < 0.001$
346 nm	0.752±0.002	0.639±0.001** ↓	t (3) = 102.149; $P < 0.001$
418 nm	1.694±0.001	2.010±0.015** ↑	t (3) = –41.198; $P < 0.001$
544 nm	0.395±0.003	0.197±0.002** ↓	t (3) = 96.287; $P < 0.001$
578 nm	0.398±0.002	0.207±0.003** ↓	t (3) = 85.222; $P < 0.001$

*Value is significant at $P < 0.05$; ** Value is significant at $P < 0.001$

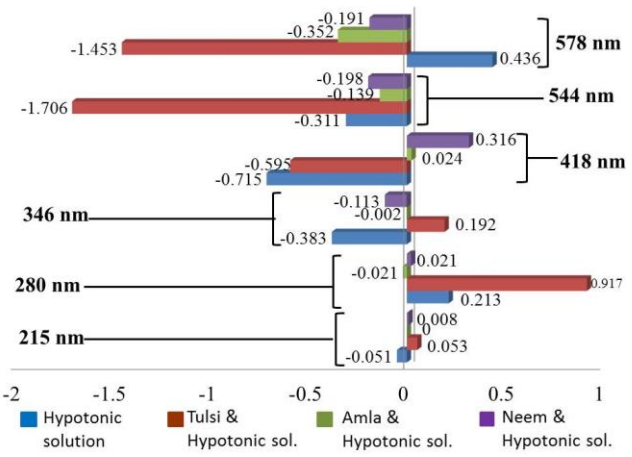

Fig. 1 — Differences in the OD values at 0 and 30 min after incubation of blood with various leaf extracts and hypotonic solution

values than those observed in the blood incubated with Neem, Amla and Tulsi leaf extracts. After 30 min of incubation, the OD values were found to be decreased in control (p=0.04), Neem (p=0.001) and Tulsi (p=0.001) treatments. However, there was no difference in the OD values after incubation with Amla leaf extract after 30 min. Thus, in blood incubated with Neem and Tulsi leaf extracts, there was much less protein breakdown as compared to control.

The OD of blood at 280 nm corresponds to the concentration of the amino acids in the sample. After incubation of blood with Neem and Tulsi, the OD values increased while with Amla the OD was found to be lowered as compared to control. In control, the OD values were found to be significantly (P <0.001) increased after 30 min of incubation while they were lowered in Neem (P <0.001) and Tulsi (p=0.004) treatments. However, there was no difference in the OD values after 30 min with Amla leaf extract.

The OD of blood at 346 nm corresponds to the concentration of the NADH and NADPH in the sample. The ODs of the test samples were much lowered as compared to control and the lowest OD was observed for blood incubated with Amla. The ODs in control and Neem treatment were significantly decreased (P <0.001) after 30 min of incubation with hypotonic solution, while with Tulsi, the OD was increased (P <0.001). However, there was no difference in the OD with Amla treatment.

The ODs of blood at 418, 544 and 578 nm correspond to the concentration of the carboxy-hemoglobin (CO-Hb) and oxy-Hb in the sample. The OD values were found to be increased in the Tulsi and Amla treatments while the OD values were generally lowered in Neem treatment as compared to control. The OD values decreased significantly (P <0.001) in control, Neem and Tulsi after 30 min of incubation with Tulsi treatment showing the steepest decline in the OD values.

In the current study, the protein levels were much reduced in test samples than in the control. This indicates that the hypotonic solution breaks down the cellular components in the blood while the leaf extracts protected the integrity of the cell membranes thereby reducing the cellular breakdown. With passage of time i.e. after 30 min of incubation, the cellular breakdown decreased in control but it increased in Neem and Tulsi treatments which indicates that the constituents in the extracts continued to act on the cells leading to their breakdown. However, in Amla treatment the protein levels did not change after 30 min which suggests that Amla was most effective in protecting the cells from breakdown.

The amino acid levels in the Neem and Tulsi treatments were found to be increased as compared to control in general and after 30 min of incubation the amino acid levels increased significantly in Tulsi treatment. It reveals higher protein catabolism due to active constituents present in the Tulsi extracts. However, the amino acid levels were lower in Amla treatment than in control suggesting that Amla extract did not facilitate protein catabolism. It is well known that normally there are many amino acids which are present in abundance in the RBCs in humans while the rest are present in low levels inside the RBCs[30]. Further, since the RBCs in chicken do not have organelles except nucleus and mitochondria, hence they can't synthesize proteins from amino acids but they contain a large pool of free amino acids, which are used for inter-organ transport[31]. Thus, increase or decrease in the amino acid levels indicates the status of protein metabolism in blood.

NADH and NADPH levels are an indication of the carbohydrate metabolism[29]. Both NAD and NADP are the coenzymes which assist in the activity of dehydrogenases and in the process NAD is converted to NADH and NADP to NADPH. The energy needs of the RBCs are fulfilled by glucose metabolism which occurs via two major pathways: glycolysis in RBCs which requires NAD^+ and the hexose monophosphate shunt that uses $NADP^+$[20]. In our study, the levels of

NADH and NADPH were found to be lowered in test samples as compared to control, lowest being for Amla treatment, suggesting lower carbohydrate metabolism in test samples. The higher carbohydrate metabolism in control might be to meet the energy demands for maintaining the cellular homeostasis due to exposure to hypotonic solution. After 30 min of incubation, the levels decreased significantly in control but increased in Tulsi treatment suggesting there might be higher carbohydrate metabolism in RBCs to provide energy for maintaining the homeostasis which might have been misbalanced due to action of active constituents in Tulsi extract. This observation is in compliance with the antidiabetic effect of Tulsi[20]. The antidiabetic effect of Tulsi is primarily due to the presence of eugenol, a major bioactive metabolite which acts to lower blood glucose level[32,33]. NADPH plays another important role as it is a cofactor in the reactions that defend RBCs against oxidative damage. The levels of NADH and NADPH were found to be lowered in test samples as compared to control. If the levels of NADH and NADPH are low or absent, the heme-iron exists in reduced form and can be oxidized to form methemoglobin which gives rise to free radicals that can cause oxidative cellular damage by damaging macromolecules like DNA, lipids and proteins[34]**.** However, after 30 min of incubation, levels of NADH and NADPH increased only in Tulsi treatment suggesting that there was less oxidative damage in the blood and the constituents in Tulsi leaf extract might have protected the cells from redox stress. Further, NADPH also regulates the levels of reduced glutathione (GSH) in RBCs which act as oxidant scavengers[35].

The absorptions at 418, 544 and 578 nm correspond to α and β peaks of oxy-hemoglobin[36] and are due to *d-f* transition of carboxy-Hb to oxy-Hb (CO-Hb to Oxy-Hb)[29]. In the current study, the levels of CO-Hb and oxy-Hb were found to be much higher in Tulsi followed by Amla treatments while they were comparatively lower in Neem treatment as compared to control. The CO is a bye product of normal metabolism i.e. catabolism of heme containing proteins or hemoglobin in RBCs[37]. This CO is not just a toxic bye-product but has been known to mediate several processes like respiration, neural signalling and blood pressure in humans[38,39]. The more the RBCs are destroyed, the more is the endogenous production of the CO due to heme catabolism involving heme oxygenase[40]. The higher levels of CO-Hb and oxy-Hb in Tulsi treated blood as compared to control indicates higher heme catabolism as a result of hemolysis and that the RBCs took up more oxygen. In a study on humans, it was observed that children with hemolytic diseases had higher levels of CO-Hb as compared to their corresponding environmental controls[41]. Further, Tulsi treatment showed the greatest decrease in the levels of CO-Hb and oxy-Hb while there was no significant change in the levels of CO-Hb and oxy-Hb in Amla and Neem treatment after 30 min of incubation *in vitro*. It is possible that Tulsi extract has prevented hemolysis and/or heme catabolism and thus reduced the endogenous production of CO. There are no studies available on relationship between plant leaf extracts and changes in the CO-Hb, oxy-Hb, NADH and NADPH levels in blood, and in this study we made an attempt to demonstrate these effects on blood *in vitro*.

Conclusion

In the blood exposed to hypotonic solution, there was cellular degradation and higher protein metabolism as was evident from the ODs of protein and amino acids; lower NADH and NADPH levels suggest lower glucose metabolism and oxidative cellular damage while higher oxy-Hb levels indicate higher oxygen absorption as compared to test samples. Among the three extracts studied, it was observed that the Amla leaf extract treatment had comparatively more protective role for cellular components of the blood as against hypotonic solution. Further, Tulsi extract was observed to significantly reduce hemolysis, heme catabolism and redox stress in RBCs. Tulsi also increased carbohydrate metabolism indicating its antidiabetic property. Thus, the results of this study have shown that Amla extract could significantly reverse the overall effects of the hypotonic solution on the cellular components while Tulsi extract showed a more protective role in maintaining homeostasis of cellular components in blood.

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Conflicts of interest

Authors declare no conflict of interests.

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