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Immunomodulatory and antioxidant activities of fresh juice extracts of *Brahmi* and *Guduchi*

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Medicinal plants mentioned in *Ayurveda* can be used as food or medicine due to their impact on human health and disease prevention. For example, *Guduchi* has been used as an immunomodulator for its ability to enhance the immune response. In the present study, fresh juice extracts of *Brahmi* and *Guduchi* was evaluated for its immunomodulatory and antioxidant activity. Fresh juice of *Brahmi* and *Guduchi* was prepared and lyophilized. The antioxidant activity of the same was evaluated against free radicals whereas immunomodulatory activity was carried out in cyclophosphamide induced immune-suppressed *Swiss albino* mice. Haemagglutination test was used to assess their effects on humoral response. Both these extracts showed *in vitro* antioxidant activities. *Brahmi* exhibited higher TAC (22.39±1.39), phenolic content (24.93±1.27) and hydroxyl radical scavenging effect (83.79 ± 0.88). Similar effects were observed with both extracts in total antioxidant activity against DPPH radical, reducing power and NO radical. Both the plants stimulated the humoral immune response. Increased haemagglutination inhibition was observed with *Brahmi* (6.40±0.24) in comparison to *Guduchi* (6.20±0.37). The results suggest that *Brahmi* and *Guduchi* both can be considered as promising immunomodulatory agents.

Keywords: Immunomodulator, Antioxidant, Cyclophosphamide, Levamisole, *Brahmi*, *Guduchi*.

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TAC: total antioxidant capacity, DPPH: 2,2-diphenyl-1-picrylhydrazyl, TCA: trichloro acetic acid, NO: nitric oxide, TPC: total phenolic content, FCR: Folin-Ciocalteu reagent, RH: relative humidity, rRBCs: rabbit red blood cells, CP: Cyclophosphamide, i.p: intraperitoneal, HA titer: haemagglutination titer, WBC: white blood cells, RBC: red blood cells.

Immunomodulators are the agents that either suppress or stimulate the immune system of the host to regulate/normalize it. They act as biological response modifiers and ameliorate the immune system that protects us against infections and foreign substances[1]. Extensive studies have been done and various synthetic agents are used for immune-suppression (such as azathioprine, 6-mercaptopurine, methotrexate and calcineurin inhibitors) or immune-stimulation (interferon alpha). But, prolonged use of these agents is often associated with adverse effects or risk of infection[2,3]. Therefore, alternative therapeutic strategies to improve the immune response without having any side effects is needed in current scenario.

From last few decades, medicinal plants have attracted much attention in the field of Pharmacology and drug discovery. Plants mentioned in *Ayurveda* have been used as a traditional remedy in several parts of the world to strengthen the immune system[4]. Studies have shown immunomodulatory activities of many plants such as *Andrographis paniculata* (Burm.f.) Nees*, Azadirachta indica* A.Juss., *Boerhaavia diffusa* L., etc.[5]. In *Ayurveda, Tinospora cordifolia* is considered as a *rasayana* that boost the immune function[6]. *Tinospora cordifolia* is commonly known as *Guduchi* (*Marathi*), belongs to family *Menispermaceae*[7]. *Guduchi* is reported to possess antispasmodic, antidiabetic, antiperiodic, antioxidant, antistress, antileprotic, antidiarrhoeal, immunomodulatory, dysentery and antipyretic activities[7,8]. The immunomodulatory activity of *guduchi* is evaluated in many studies through preparing its aqueous extracts (*satwa*), and ethanolic extracts[6,9,10].

Similarly, *Bacopa monneri* belonging to family *Scrophulariaceae*, commonly known as *Brahmi*

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(*Hindi*) is another such plant which has been used for many years as a memory enhancer. Various pharmacological studies have demonstrated analgesic, antipyretic, anti-inflammatory, sedative, antiepileptic, antidepressant, antineoplastic and calcium antagonist activities of *Brahmi*[11-13].

In *Ayurveda*, according to *Pancha-Vidha Kasaya Kalpana*, the most potent extract of any plant is its *Swaras,* i.e., fresh juice[14]. The present study is designed to evaluate the antioxidant and immunomodulatory activity of dried juice extracts of *Brahmi* and *Guduchi*. It is of utmost importance to recognize the most potent preparation of *Guduchi* and *Brahmi* to improve their efficacy as an immunomodulator. Stimulation of the immune response can prevent various infectious diseases and allergies[15]. Therefore, evaluation of medicinal plants such as *Brahmi* and *Guduchi* that can be used as a dietary herb and stimulates the immunity should be considered as new forms of treatment. In the present study, the immune-stimulatory and antioxidant potential of these two dietary herbs was evaluated.

Methodology

Collection of plant materials

Plant materials were collected from the farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow and authenticated by the department of Botany CSIR-CIMAP, Lucknow. Specimens of the plants collected were preserved in Herbal Medicinal and Products Department, CSIR-CIMAP, Lucknow (HMPD/PHE-01 for Brahmi & HMPD/PHE-02 for *Guduchi*).

Preparation of *Swaras*

Five hundred gm of fresh stems of *Guduchi* and whole plant of *Brahmi* were taken separately into Juicer Mixer Grinder (Philips). Plant material was crushed in grinder about 10 min till a thin paste was obtained. The juice was filtered, concentrated on a rotary evaporator (Buchi R210, Switzerland) and subjected to lyophlization (Labconco, Bio Gen Tek). Obtained dried juice extracts were stored in airtight container for further study. The yield of *Guduchi* and *Brahmi* was 6.35 % and 9.17 %, respectively.

Phytochemical analysis

Preliminary phytochemical analysis was performed in *swaras* of both the plants following the standard methods[16].

Antioxidant activity

Total antioxidant capacity (TAC) estimation

Falleh *et al*., method was used to determine the total antioxidant capacity[17]. 100 µl of different concentrations of samples (10~200 µg/mL) were reacted with 1 mL TAC reagent (0.3 N sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Samples were incubated on a water bath at 95 ^{0}C for 90 min. After cooling the samples to room temperature, the absorbance was taken at 695 nm with the help of UV spectrophotometer (Shimadzu 1601 UV–VIS Spectrophotometer, Japan). Milli Q water (Millipore, Bedford, MA, USA) mixed with the reagent and incubated under same condition was used as blank. The antioxidant activity is expressed as the number of equivalents of mg gallic acid per gram dry weight.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

The free radical scavenging activity of plant juice extracts of *Brahmi* and *Guduchi*, was carried out using a method described by Yen GC *et al*. with slight modification[18]. 100 µL of the DPPH solution (0.1 M in methanol) was added to 400 µL of different concentrations of *Brahmi* and *Guduchi* extract (10, 25, 50, 100 and 200 µg/mL). The mixture was shaken and incubated under dark for 30 min at room temperature. Absorbance was taken at 517 nm. The percentage inhibition was calculated by using an equation:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where, A_0 is absorbance of the control, A_1 is absorbance of extracts/standard.

Reducing power estimation

The reducing power of *Brahmi* and *Guduchi* was estimated following the method of Rainha *et al*.[19]. 200 µL of each sample was mixed with 200 µL Phosphate Buffer (300 mM, 6.6 *p*H) and 200 µL Potassium Ferricyanide (1 % w/v). The mixture was incubated on a water bath at 50 ^{0}C for 20 min. The mixture was cooled at room temperature, followed by the addition of 200 µL of Trichloro acetic acid (TCA, 10 % w/v). The mixture was centrifuged at 3000 rpm for 5 min to collect the 100 µL upper layer of the solution. The collected upper layer was mixed with 100 µL double distil water and 20 µL of $FeCl_3$ (0.1 % w/v) and absorbance was taken at 700 nm against blank.

Nitric oxide radical scavenging activity

Two hundred µL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer saline (*p*H 7.4) is mixed with 25 µL of sample at various concentrations (10~200 µg/mL). The mixture was incubated at room temperature for 150 min. 50 µL of the incubated solution was withdrawn and mixed with 100 µL Sulfanilamide (1 % in 5 % Phosphoric acid) and incubated for 5 min at room temperature. 100 µL of 0.1 % (α-napthyl)-ethylene diamine was added to the reaction mixture and again incubated at room temperature for 30 min. Absorbance was measured at 546 nm. IC_{50} value was calculated by using formula:

$IC_{50} = (\sum C/\sum I) * 50$

Where, ∑C is the sum of extract concentrations used to test and ∑I is the sum of the % of inhibition at different concentrations[20].

Hydroxyl radical scavenging activity

Fifty µL sample was mixed with 50 µL of $FeSO_4.7H_2O$ (10 mM), EDTA (10 mM), 2-deoxyribose (10 mM) and 250 µL of phosphate buffer (0.1 M, 7.4 *p*H). 50 µL of H_2O_2 (10 mM) was added into reaction mixture and incubated at 37 °C for 4 hrs. Finally, 250 µL each of TCA (2.8 %) and Thiobarbituric acid (1 %) were added into the incubated mixture and the resultant solution was boiled for 10 min in a water bath, cooled in ice and absorbance was measured at 520 nm[21].

Total phenolic content (TPC) estimation

TPC of plant juice extracts of *Brahmi* and *Guduchi* was determined with the help of Folin-Ciocalteu reagent[22]. 10 µL samples were mixed with 100 µL FCR (10 % v/v) and 80 µL Sodium carbonate (7.5 %). The mixture was incubated at 40 ^{0}C for 30 min. Absorbance of all samples was measured at 765 nm. Total phenolic content expressed as number of equivalents of mg gallic acid per gram using the equation obtained from a standard gallic acid calibration curve.

Immunomodulatory activity

Experimental animals

Swiss albino mice weighing 30-35 gm were used in the study. They were acclimatized under standard laboratory condition (22 ± 5 ^{0}C and 55 ± 5 % RH) one week prior to the experiment. All the mice received standard diet and water *ad libtum* and maintained under 12 hrs light/dark cycle. The experimental protocol was approved by the Institutional Animal Ethics Committee of CSIR-CIMAP, Lucknow (AH 2012-11). The study was carried out in accordance with CPCSEA guidelines.

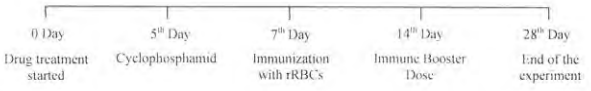
Preparation of antigen

Blood was collected from New Zealand rabbit through the central artery of the ear in heparin containing centrifuged tube. Blood was centrifuged at 2000 rpm for 10 min at 4 ^{0}C temperature and the supernatant was discarded. Pellets containing rRBCs were suspended in equal volume of Alsever's solution (1:1). Pellets were washed thrice with an Alsever's solution. The rRBCs ($2x10^8$ cells/mL) was suspended in normal saline for immunization[23].

Treatment protocol and immunization schedule

The mice were divided into five groups (n = 6). Group-I (vehicle control) and II (negative control) received distilled water daily 10 mL/kg body weight, Group-III (positive control) received Levamisole at 0.68 mg/kg *per os* dose[24]; Group-IV and V received *Brahmi* and *Guduchi* at 400 mg/kg dissolved in water, *per os* dose[25,26]. All the animals were treated with respective drug dose for 28 days. On day 5th all the animals except group I were treated with CP 200 mg/kg/i.p.

On the 7th day of the treatment with drugs, all the groups except group I were immunized with 200 µL of $2x10^8$ cells/mL rRBCs (in 10 % normal saline) i.p. Again on the 14th day similar dose of rRBCs was given as an immune booster dose. On 28th day, 0.5 mL blood sample was collected from the retro-orbital plexus with the help of hematocrit capillaries (Himedia, Mumbai, India). Serum was separated and kept in deep freezer (Vestfrost, at -20 ^{0}C) until use[23].



HA titer assay

The antibody levels were determined by HA titer technique. Serial two fold diluted serum in Alsever's solution (100 µL) was mixed with 100 µL rRBCs (10 % in normal saline) in microtiter plate (96 well plate) (Axygen Life sciences, California). They were allowed to incubate at room temperature for 1-3 hrs and examined visually for agglutination. rRBCs setting patterns was read. Highest serum dilution value showing visible agglutination was taken as antibody titer. The HA titer was expressed as the reciprocal of the heist dilution of the serum showing definite agglutination formation (positive pattern)

compared with the smooth dot in the center of the well (negative pattern)[23].

Hematological assay

The effects of *Brahmi* and *Guduchi* on WBC and RBC count were examined by using blood samples collected on the 28th day with the help of hemocytometer (Rohem, New Delhi, India).

Acute toxicity study

Acute toxicity of the fresh juice extracts of both the plants were performed according to the OECD (Organization of Economic Cooperation and Development) Guideline 423[27].

Statistical analysis

The effect of juice extracts on the HA titer test and other parameters were compared with the control by using one-way analysis of variance with Dunnett's post hoc test (GraphPad Instant®) and Tukey's multiple comparison tests using Graph Pad Instat®. The value of significance was fixed at $p < 0.05$. Values are expressed as mean ± Standard error of mean (SEM).

Results and discussion

As mentioned earlier, *Brahmi,* a well known memory enhancer exhibits various important ethno-pharmacological uses against various diseases such as anti-inflammatory, antidepressant, antimicrobial, hepatoprotective, etc. In *Ayurveda,* the most potent extract in terms of therapeutic efficacy is the fresh juice which is nontoxic to humans and devoid of toxic solvent. *Guduchi* is referred to as a *Rasayana*, which is a known immunomodulatory agent in *Ayurveda* and its activity is also supported by several studies[5]. However, the immunomodulatory activity of its juice has not been evaluated yet. Investigation of fresh juice extract of *Brahmi* and *Guduchi* will provide a scientific evidence for these plants to be used as a dietary herb that will help in various disease prevention.

Phytochemical screening

The qualitative phytochemical screening of the dried juice of *Brahmi* and *Guduchi* confirmed the presence of alkaloids, glycosides, cardiac glycosides, terpenoids, flavonoids, steroids, tannins, and saponins (Table 1).

Table 1 — Phytochemical screening of dried juice of both plant.

Phytochemical test	*Brahmi*	*Guduchi*
Test for alkaloids		
Dragendroff's test	+	+
Wagner's test	+	+
Test for cardiac glycosides		
Keller-Killiani test	+	+
Test for flavonoids		
Shinoda Test	+	+
Test for phenolics		
$FeCl_3$ test	+	+
Test for terpenoids		
Salkowski test	+	+
Test for Saponin		
Foaming test	+	+
Test for steroids	+	+
Test for tannins	+	+

Antioxidant activity of the dried juice extracts

Free radicals play an important role in various pathological diseases. In a cellular system, the ROS (reactive oxygen species) is responsible for cell damage and also for cell death[28]. Antioxidant inhibits the formation of free radical by reducing the ROS or form chelate itself with ROS[29]. Various antioxidant methods have developed for estimation of antioxidant activity and to explain how antioxidants work. The total phenolic content, total antioxidant capacity, total flavonoid content, reducing power, DPPH, NO, hydroxyl radical scavenging activity estimation is the most common methods for evaluation of the antioxidant activity of plant extract[30]. The juice extracts of *Brahmi* and *Guduchi* were tested for their antioxidant capacity. The TAC of the plant juice extracts of *Brahmi* and *Guduchi* increased with increasing concentration (Fig. 1a). At 200 µg/mL, the antioxidant capacities of both plant juice extracts were similar with no statistical difference.

DPPH is an unstable free radical, easily accept the electron or hydrogen and become to stable. DPPH having deep purple color in methanol solution and showing maximum absorbance at 517 nm. In the presence of an antioxidant deep purple color was changed into yellow color due to scavenging of free radicals[31,32]. The free radical scavenging activity of both extracts was expressed in terms of % inhibition of DPPH radical. All the concentrations of the test solution more or less inhibited the free radical as shown in Fig. 1b. IC_{50} of *Brahmi* and *Guduchi* were found to be 56.60 µg/mL and 60.91 µg/mL, as compared to Gallic acid used as a standard (IC_{50} of 46.08 µg/mL).

Brahmi and *Guduchi* have shown very less reducing activity in comparison to gallic acid

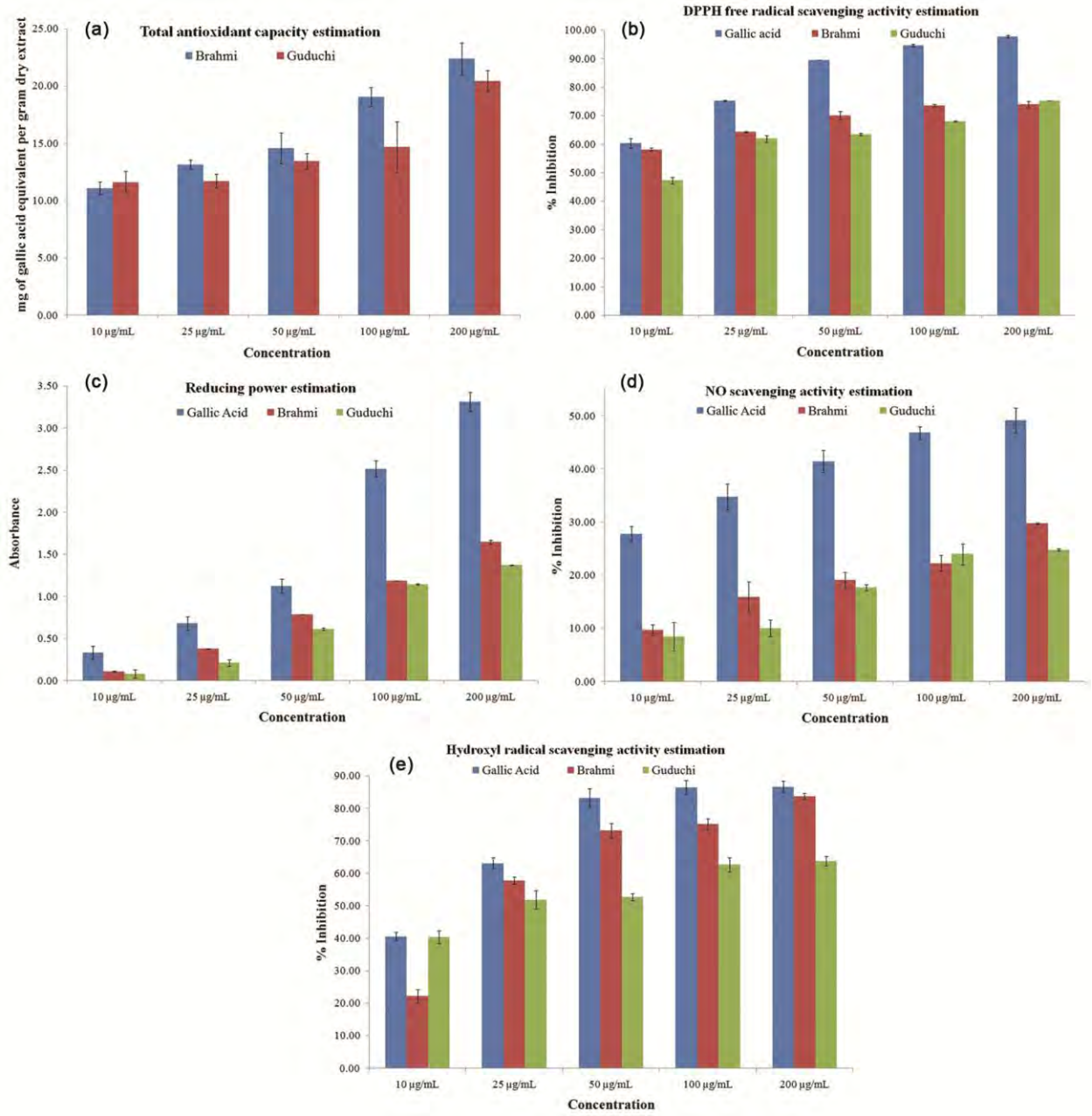


Fig. 1 — *In vitro* antioxidant activity of fresh juice of Brahmi and Guduchi: (a) Total antioxidant capacity, (b) against DPPH radical, (c) Reducing power determination, (d) against NO radical, (e) against hydroxyl radical.

(Fig. 1c). Similarly, both the extracts (IC_{50} of *Brahmi* 198.72 µg/mL and *Guduchi* 226.23 µg/mL) scavenge a lesser amount of the nitric oxide radical in comparison to the gallic acid (96.25 µg/mL) used as a standard (Fig. 1d). Hydroxyl radical scavenging activity was studied by estimating hydroxyl radical induced deoxyribose degradation (non-site specific) using the thiobarbituric acid method. The complex was formed by interaction between EDTA and iron (III) in solution by which hydroxyl radicals were produced. Hydroxyl radical formation will be terminated if the extract having chelating property as well as preventing deoxyribose from hydrogen peroxide[33]. All the extracts showed scavenging activity against hydroxyl radical in a concentration dependent manner. The highest % inhibition was obtained with *Brahmi* (Fig. 1e). IC_{50} of *Brahmi* 61.62 µg/mL, *Guduchi* 70.89 µg/mL and gallic acid 53.42 µg/mL. An increase in the absorbance shows an increase in the antioxidant activity (Fig. 2). The

phenolic content of the plant extract is responsible for antioxidant activity[34]. Perhaps, the antioxidant activity of the fresh juice extracts of *Brahmi* and *Guduchi* is related to total phenolic content which when determined was found to be 24.93 and 24.17 mg of gallic acid equivalent per gram dry weight, respectively. These results show that fresh juice extracts of *Brahmi* and *Guduchi* are promising source of antioxidants.

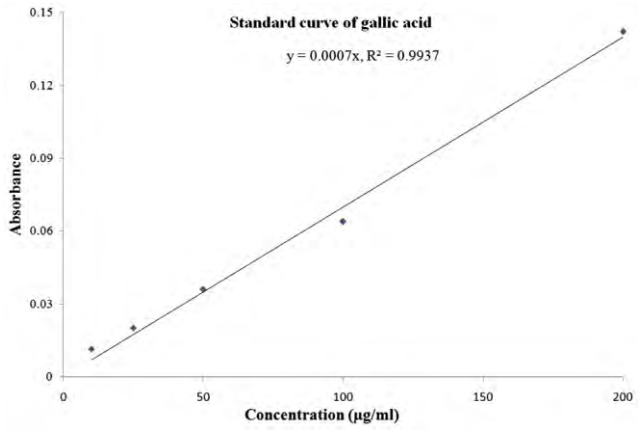


Fig. 2 — Standard curve of gallic acid

Immunomodulatory activity

Immune system is an important system in the body that protect us against various pathogens and foreign bodies. In particular, humoral immune response plays a major role by preventing the intracellular infections through production of antibodies[35]. Haemaglutination inhibition is often used to determine the humoral response. The summary of the result of immunomodulatory (HA titer) test is shown in Table 2. Significant increase was observed in HA titer of animals treated with *Brahmi* (6.40 ± 0.24) and *Guduchi* (6.20±0.37) when compared to the negative control group (2.60 ± 0.40). The augmentation of humoral immune response to rRBCs is clearly indicated by both these extracts and standard drug, Levamisole. HA titer indicates the level of immunoglobulin produced which are mainly responsible for activation, opsonization and neutralization of toxins[6]. The data suggests that *Brahmi* and *Guduchi* both possess immunomodulatory activity and are safe as depicted in acute oral toxicity study. Therefore, these dietary herbs can be used as an immunomodulatory agent. Although the exact mechanism by which these extracts modify the immune response is not yet known and can be explored in future studies.

Table 2—Effect of dried juice on hematological parameters

Groups	HA titer	WBCs count(in 10^3/mm^3)	RBCs count (in 10^6/mm^3)
Vehicle Control	4.40 ± 0.68	13.45±0.44	5.93±0.53
Cyclophosphamide	2.60 ± 0.40	10.49±0.48	3.79±0.71
Levamisole	7.00 ± 0.55***	21.51±0.72***	6.88±0.67***
Brahmi	6.40 ± 0.24***	29.48±0.59***	7.77±0.82***
Guduchi	6.20 ± 0.37***	26.08±0.40***	6.48±0.93***

n=6; values are represented as mean ± SEM., ***=p<0.001.

Table 3 — Effect of dried juice of both plants as a single acute oral dose on body weight, haemoglobin and serum biochemical parameters in *Swiss albino* mice

Parameters/ Groups	Control	*Brahmi* (2000 mg/kg)	*Guduchi* (2000 mg/kg)
Body weight (gm)	24.5±0.76	24.33±1.26	24.83±0.65
Haemoglobin (gm/dL)	11.29±0.84	12.25±0.73	10.67±0.54
SGOT (U/L)	25.27±1.32	27.67±1.26	31.34±4.20
SGPT (U/L)	22.28±3.73	23.15±5.22	22.52±1.69
ALKP (U/L)	208.61±12.43	227.47±25.91	231.14±12.24
Bilirubin (mg/dL)	0.40 ± 0.10	0.60 ±0.19	0.52 ± 0.10
Cholesterol (mg/dL)	80.61±4.26	75.44±4.08	59.82±1.96
Triglycerides (mg/dl)	121.45±24.02	106.46±13.63	127.23±18.15
LDL (mg/dL)	79.77 ± 3.76	86.53 ± 4.21	91.92 ± 4.40
Creatinine (mg/dL)	0.63±0.18	0.59±0.24	0.45±0.29
Blood urea (mg/mL)	31.11±0.14	36.88±0.13	28.94±0.10

n=6; values are represented as mean ± SEM.

Acute toxicity study

In acute toxicity study, oral administration of fresh juice extracts of *Brahmi* and *Guduchi* at 2000 mg/kg did not produce any signs of toxicity. All the animals were alive and no significant change was observed in biochemical parameters as compared to the control group (Table 3).

Conclusion

In the present study, fresh juice extracts of *Brahmi* and *Guduchi* was evaluated for the first time with respect to their antioxidant and immunomodulatory activity. Fresh juice extracts show high antioxidant activities as well as immunomodulatory activities. The fresh juice extracts therefore can be used as a dietary herb in clinical applications. Furthermore, the study point to a new possibility of using the dietary herbs as a therapeutic agent.

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Conflicts of interest

There are no conflicts of interest.

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