

# Antioxidant and anti-inflammatory properties of G-immune plus: A polyherbal formulation

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## Abstract

**Background:** G-Immune Plus is a polyherbal formulated capsule made from the extracts of *Wedelia calendulacea*, *Tinospora cordifolia*, *Withania somnifera*, *Centella asiatica*, *Emblica officinalis*, *Asparagus racemosus*, and *Tribulus terrestris*. **Materials and Methods:** The antioxidant properties of the polyherbal formulation G-immune plus capsule were evaluated by 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay, 2, 2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay, total antioxidant capacity assay, ferric reducing antioxidant power assay, and copper reducing power capacity assay. Likewise, the anti-inflammatory properties were evaluated by inhibition of albumin denaturation assay and membrane stabilization assay. **Results and Discussion:** The antioxidant capacity and reducing activities by the antioxidants present in the formulation were observed proving the presence of antioxidant activity. The radical scavenging activity was observed with the IC<sub>50</sub> value being 7.49 mg/ml from ABTS assay. The anti-inflammatory activity by stabilizing the membrane was found with the IC<sub>50</sub> value being 7.02 mg/ml from membrane stabilization assay. **Conclusion:** The present study suggested that this polyherbal formulated capsule possess moderate to potential antioxidant and anti-inflammatory activities. Due to the presence of multiple herbs with enormous therapeutic activities within this formulation, the capsule could also exert multiple pharmacological effects on its targets and making it a potent Ayurceutical supplement for human health.

**Key words:** Anti-inflammatory, antioxidant, ayurceutical, polyherbal, synergy, *Wedelia calendulacea*

## INTRODUCTION

Free radicals are unstable molecules that have lost an electron in its valence shell. They can attack any DNA, leading to dysfunction, mutation, and cancer, etc. They can also target the enzymes and proteins, disturbing the normal cell activities, or cell membranes, leading to a chain reaction of denaturation.<sup>[1]</sup> Antioxidants both endogenous and exogenous are used to neutralize free radicals and protect the body from the attack of free radicals by maintaining redox balance.<sup>[2]</sup>

Inflammation is our body's response to outer threats such as infection, stress, or toxic chemicals, where our immune system responds by initiating proteins meant to protect cells and tissues. Moreover, this can be sometimes harmful to our own body leading to multiple diseases. Thus, for controlling and inhibiting this inflammatory issues steroid, nonsteroid anti-inflammatory drugs are used. However,

these are associated with adverse effects, creating a need for an alternative drug which on minimum effective dosage exhibits the highest efficacy with the least adverse effects. Thus, the use of herbal plant extracts containing anti-inflammatory activities is being used in recent days.<sup>[3]</sup>

G-immune plus is a polyherbal formulated capsule containing extracts taken from various parts of 7 herbal plants possessing antioxidant and anti-inflammatory properties Table 1. These 7 herbal plants are: *Wedelia calendulacea*, *Tinospora cordifolia*, *Withania somnifera*, *Centella asiatica*, *Emblica officinalis*, *Asparagus racemosus*, and *Tribulus*

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*terrestris*. This herbal capsule is prescribed for improving the immunity of humans and to improve the general health. In this modern environment, there is a huge demand for intake of antioxidant and anti-inflammatory foods or dietary supplements with the hope of maintaining body healthy and free from diseases. Thus, this herbal formulation can provide the much-needed solution required to the people for leading a healthy life.

## MATERIALS AND METHODS

### Sample Preparation

The capsule containing the polyherbal extract was taken and weighed appropriate quantity to prepare a stock solution and dissolved with water. The solution was stirred well for 2 h using magnetic stirrer. The mixed solution was filtered using filter paper and stored in refrigerated condition for further use.

### *In vitro* Antioxidant Assays

#### Total antioxidant capacity (TAC) assay

The assay is based on the formation of green phosphate-molybdenum complex by the process known as reduction, where molybdenum (VI) is converted to molybdenum (V) if there is the presence of an antioxidant in the extracts at acidic pH which is measured at 695 nm. The samples were prepared at different concentrations (2 mg/ml - 10 mg/ml). 1 ml of reagent (28 mM disodium hydrogen phosphate + 4 mM ammonium molybdate + 0.6 M sulfuric acid) was added to 0.1 ml of test samples and was incubated at 95°C for 90 min in boiling water bath.<sup>[4]</sup> The tubes were then cooled to room temperature, and the absorbance was measured at 675 nm. Ascorbic acid was used as standard, and the results were expressed as ascorbic acid equivalents/g extract.

#### 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay is based on the reduction of DPPH by the scavenging activity of antioxidants present in the extracts by the donation of protons. This was indicated by the change of color from purple to yellow color which was measured by a decrease in absorbance at 517 nm. 0.5 ml of DPPH reagent was added to 2 ml of test samples prepared in different concentrations (100–500 µg/ml) using methanol. Methanol with DPPH used as control and methanol alone as blank.<sup>[5]</sup> Results were expressed as the percentage reduction of the initial DPPH absorption in relation to the control. The concentration of extract leading to 50% reduction of DPPH (IC<sub>50</sub>) was also determined.

Formula for finding out the percentage inhibition:  $[(\text{OD of control} - \text{OD of test})/\text{OD of control}] \times 100$  (Eq: 1)

Where OD represents the optical density or absorbance. The IC<sub>50</sub> values were calculated from the graph plotted as inhibition percentage against the concentration.

#### 2, 2'-azinobis-3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The ABTS radical scavenging assay method was based on the conversion of the disodium ammonium salt ABTS to its radical cation by the addition of ammonium persulfate which results in the reduction of blue color which is read at 745 nm. The ABTS standard solution was prepared by mixing equal volumes of 7 mM ABTS solution and 2.45 mM ammonium persulfate and was kept for 16 h at room temperature to form a dark colored solution whose absorbance was read at 745 nm.<sup>[6]</sup> The ABTS standard solution was diluted using water to reach the final absorbance of  $0.700 \pm 0.02$ . About 1 ml of ABTS standard solution was mixed with 30 µl of the test sample prepared at different concentrations (2–10 mg/ml). The decrease in absorbance was read at 745 nm after a period of 6 min. ABTS with water was taken as control and water without ABTS as blank. Results were expressed as the percentage reduction of the initial ABTS absorption in relation to the control. The concentration of extract leading to 50 % reduction of ABTS (IC<sub>50</sub>) was also determined.

The percentage inhibition and IC<sub>50</sub> were calculated as mentioned in the DPPH radical scavenging assay.

#### Ferric reducing antioxidant power (FRAP) assay

The FRAP assay depends on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) reduction to ferrous tripyridyltriazine (Fe (II)-TPTZ) by a reductant at low pH. Ferrous (II)-TPTZ has an intensive blue color and can be monitored at 593 nm. Briefly, the FRAP reagent was prepared using an acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid, and 20 mM Ferric (III) chloride solution in proportions of 10:1:1 (v/v), respectively. 90 µl of the test sample (2–10 mg/ml) was added to 2.7 ml of the FRAP reagent, and 270 µl of water was added finally after which the reaction mixture was incubated at 37°C for 30 min.<sup>[7]</sup> The increase in absorbance of the reaction mixture was recorded at 593 nm. All the measurements were taken in triplicate, and the mean values were calculated.

#### Copper reducing power capacity (CUPRAC) assay

The CUPRAC method involves the reduction of cupric ion to cuprous ion by the presence of antioxidants in the sample. It follows the mechanism of electron transfer. 1 ml of ammonium acetate (6.166 g in 80 ml) was added to 1 ml of the test sample prepared in different concentrations (2–10 mg/ml). To this, 1 ml of cupric chloride solution (0.137 g in 80 ml) and 1 ml of neocuproine (0.125 g in 80 ml) were added after which the reaction mixture was incubated for 30 min.<sup>[8]</sup> The increase in absorbance was read at 450 nm. All the measurements were performed in triplicate, and the mean values were calculated and noted down.

## ***In vitro* Anti-inflammatory Assays**

### ***Inhibition of albumin denaturation***

The 5 ml of the reaction mixture was comprised 0.2 ml of eggs albumin (from hens egg), 2.8 ml of phosphate buffered saline (pH 6.4), and 2 ml of varying concentration (2–10 mg/ml) of extracts. Similar volume of distilled water served as a control. Then, the mixture was incubated at 37°C in BOD incubator for about 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm using pure blank. The percentage inhibition of protein denaturation and IC50 value was determined.

The percentage inhibition and IC50 values were calculated as mentioned in the DPPH radical scavenging assay.

## **Membrane Stabilization**

### ***Preparation of human red blood cell (HRBC) suspension***

Anticoagulated human blood was collected by a collection of blood in a tube containing an equal volume of alsever solution, and this was centrifuged at 3000 rpm for 10 min.<sup>[9]</sup> The obtained solution was washed thrice with isosaline solution until the supernatant becomes colorless. RBC layer was collected and diluted to make up to 10% v/v using isosaline solution.

### ***Effects of various extracts on HRBC system***

The reaction mixture (5 ml) consisted of 2 ml hyposaline (0.36% w/v NaCl), 1 ml 10 mM phosphate buffer, pH 7.4, 1 ml test sample prepared in different concentration (2–10 mg/ml), and 1 ml of HRBC suspension. For the control test, 1.0 ml of isosaline was used instead of the test sample, and hyposaline was replaced with distilled water. The mixture was incubated at 56°C for 30 min, and then the test tubes were cooled under running tap water for 10 min. The mixture was centrifuged, and the absorbance value of the hemoglobin content in the supernatant was read at 560 nm.

The percentage inhibition and IC50 were calculated as mentioned in the DPPH radical scavenging assay.

The percentage hemolysis was calculated as: (OD of test/OD of control)\*100 (Eq: 2)

## **RESULTS AND DISCUSSION**

### ***In Vitro* Antioxidant Assays**

#### **TAC activity**

The total antioxidant assay gives an estimate of the overall antioxidant potential of the components present in G-immune plus capsule. There is a formation of phosphomolybdenum complex, the intensity of which indicates the ability of the

polyherbal extracts as a scavenger of free radicals. The TAC of this polyherbal extracts was expressed as a number of equivalents of ascorbic acid.

From the Table 2, it was found that the antioxidant activity was ranging in between 2 mg/ml and 10 mg/ml concentration of the polyherbal extract where 10 mg/ml containing the highest activity.

When combining the multiple herbs in a particular ratio, it will give a better therapeutic effect and reduce the toxicity.<sup>[10]</sup> Thus, our polyherbal formulation seems to contain better antioxidant capacity than its individual components effect.

#### ***DPPH radical scavenging activity***

This free radical scavenging assay is based on the ability of the antioxidants present in the G-immune plus a polyherbal formula to decolorize the DPPH radical.

Free radical scavenging potential (DPPH) of the polyherbal extract G-immune plus at different concentrations is represented below. The radical scavenging activity increases with increase in the concentration of the sample which was reflected at the decrease in the absorbance.

From the Figure 1, it was inferred that at 500 µg/ml the sample exhibited maximum radical scavenging activity.

The IC<sub>50</sub> value was found to be 65.49 µg/ml.

This value proves that our polyherbal formulation possesses better scavenging effect than its individual monoherbal formulations at lower concentrations.<sup>[11,12]</sup>

#### ***ABTS cation radical scavenging activity***

This radical scavenging assay was used to determine the efficiency of the G-immune plus polyherbal formulation to successfully scavenge the ABTS radical. The absorbance of the sample decreases with increase in the concentration of the sample and thus increasing the percentage inhibition.

Free radical scavenging potential (ABTS) of different concentrations of G-immune plus polyherbal formulation was represented in Figure 2.

The IC<sub>50</sub> value was found to be at 7.49 mg/ml.

Compared to the DPPH scavenging activity the scavenging effect of our polyherbal formulation toward ABTS seems lower even that its monoherbal counterparts.<sup>[13]</sup> This could be due to the synergic effect of multiple components present in this formulation.

#### ***FRAP activity***

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method using an easily reduced oxidant,

Fe (III). Reduction of a ferric tripyridyltriazine complex to ferrous-(2,4,6-tripyridyl-s-triazine)<sub>2</sub> that is ferric (III) [colorless] to ferrous (II) [Blue] can be monitored by measuring absorbance at 595 nm. The absorption readings are related to the reducing power of the electron donating antioxidants present in the test compound.

The ferric reducing antioxidant potential of different concentrations of G-immune plus polyherbal formulation was represented in Figure 3.

Although the concentration level is high, the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.<sup>[14]</sup>

### CUPRAC

The chromogenic redox reagent used for the CUPRAC assay was bis (neocuproine) copper (II) chelate. This reagent works at pH 7, and the absorbance of the Cu (I) - chelate formed as a result of redox reaction with reducing polyphenols was measured at 450 nm. The color was due to the Cu(I)-Nc chelate formed, obtained with reacting varying concentrations of antioxidants with the CUPRAC reagent.<sup>[8]</sup>

This method is based on the ability of the antioxidants present in the polyherbal formulation to reduce cupric ions to cuprous ions.

The copper reducing capacity of different concentrations of G-immune plus polyherbal formulation was represented in Figure 4.

The OD value was near constant until 1 mg/ml after which there was a gradual increase in absorbance with an increase in concentration. Thus, the reducing power of this formulation ranges from 2 mg/ml to 10 mg/ml concentration level.

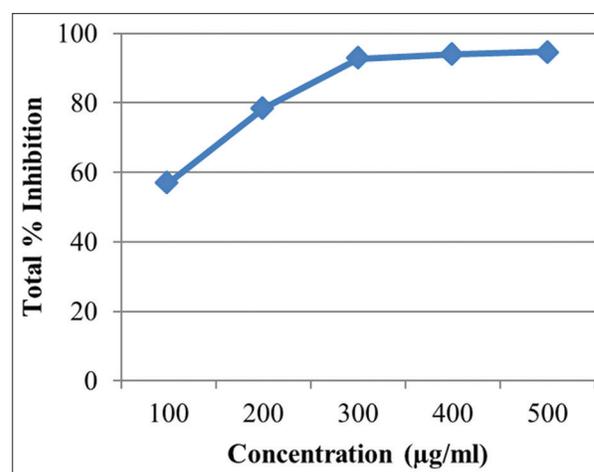
### In Vitro Anti-inflammatory Assays

#### Inhibition of albumin denaturation

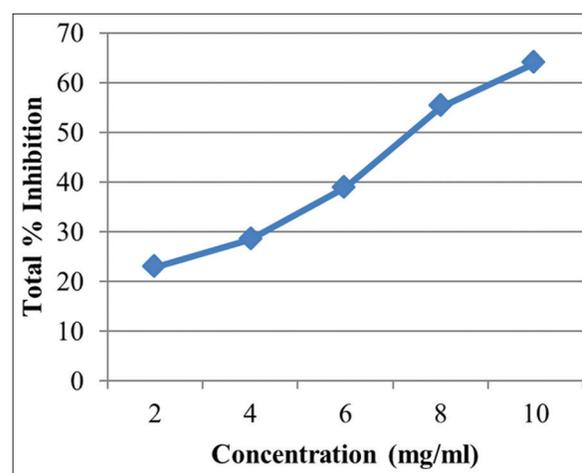
This relies on the efficiency of the extract to protect the protein from getting denatured from external and internal factors. The amount of protein denatured gets lower with an increase in the concentration of the extract showing the ability of the extract to protect the protein from the denaturation process. This inhibitory effect of protein denaturation can be confirmed by the decrease in absorbance observed in the final supernatant.

The percentage inhibition of protein denaturation by different concentrations of G-immune plus polyherbal formulation was represented in Figure 5.

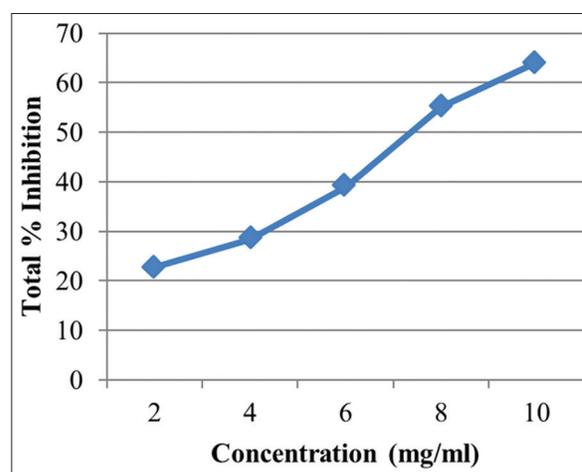
The IC<sub>50</sub> value was found to be at 13.32 mg/ml.



**Figure 1:** Scavenging effect (%) on 1,1-diphenyl-2-picrylhydrazyl radical by G-immune plus polyherbal formulation at different concentration levels



**Figure 2:** Scavenging effect (%) on 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical by G-immune plus polyherbal formulation at different concentration levels



**Figure 3:** Ferric reducing antioxidant potential by G-immune plus polyherbal formulation at different concentration levels

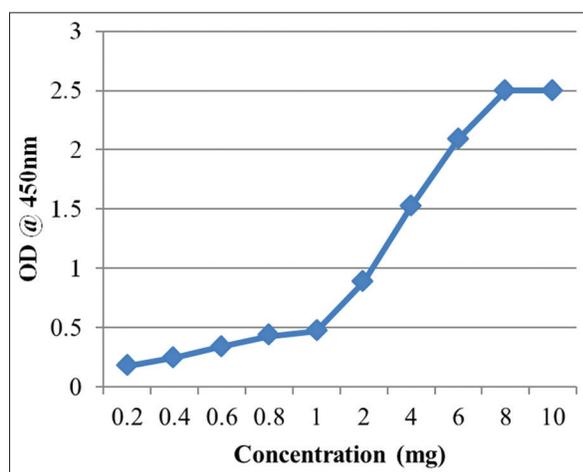
**Table 1:** Formulation of G-immune plus

Botanical name of plant	Tamil name	Each capsule contains (in mg)	Parts used
<i>Wedelia calendulacea</i>	Manjal karasilankanni	200 mg	Plant
<i>Tinospora cordifolia</i>	Seenthil kodi	100 mg	Stems
<i>Withania somnifera</i>	Amukkira	100 mg	Root
<i>Centella asiatica</i>	Vallarai	100 mg	Plant
<i>Emblica officinalis</i>	Nellikai	100 mg	Fruit
<i>Asparagus racemosus</i>	Thaneer vittan kilangu	50 mg	Root
<i>Tribulus terrestris</i>	Nerinji mull	50 mg	Plant

**Table 2:** TAC of G-immune plus capsules at different concentration levels

Test sample concentration (mg/ml)	2	4	6	8	10
AAE value ( $\mu\text{M}$ AAE/g extract)	1.01 $\pm$ 0.03	1.92 $\pm$ 0.07	2.81 $\pm$ 0.13	3.74 $\pm$ 0.05	4.84 $\pm$ 0.12

Values represent mean $\pm$ standard deviation of three replicates. TAC: Total antioxidant capacity, AAE: Ascorbic acid equivalent



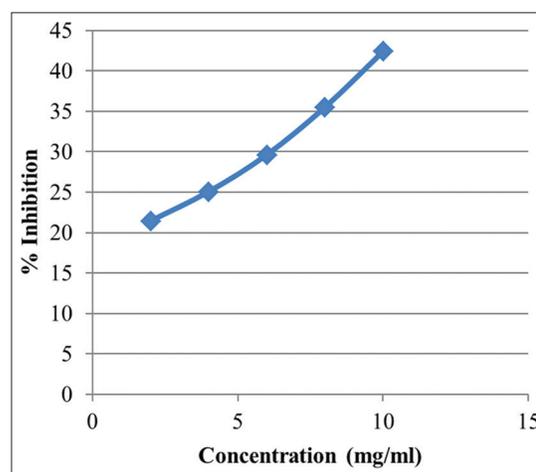
**Figure 4:** Copper reducing power capacity at different concentrations of G-immune plus polyherbal formulation

Denaturation of proteins is a well-documented cause of inflammation.<sup>[15]</sup> As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. The polyherbal formulation was effective in inhibiting heat-induced albumin denaturation. Maximum inhibition of 43% was observed at 10 mg/ml.

#### Membrane stabilization assay

The stability of the membrane gets interrupted by many external forces leading to its destabilization. This destabilization will lead to the hemolysis process which can be prevented by anti-inflammatory agents present in our polyherbal formulation. The inhibitory effect of hemolysis process can be indicated by the decrease in absorbance of the hemoglobin content present in the final supernatant.

The percentage inhibition of hemolysis by different concentrations of G-immune plus polyherbal formulation was represented in Figure 6.



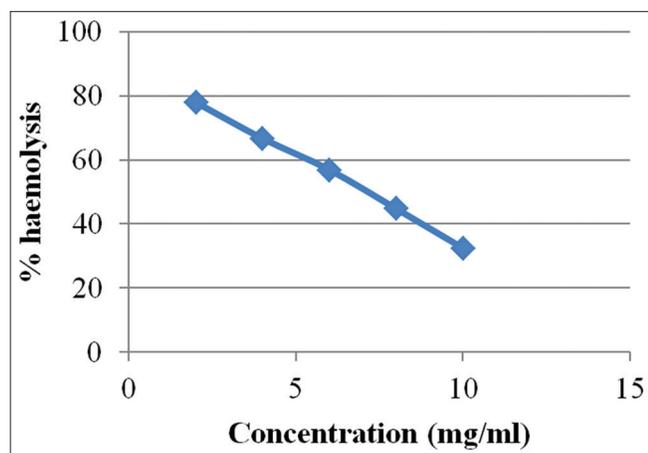
**Figure 5:** Inhibition effect (%) on albumin denaturation by G-immune plus polyherbal formulation

The IC<sub>50</sub> value was found to be at 7.02 mg/ml.

It showed the maximum inhibition of 67.51  $\pm$  3.36% at 10 mg/ml. With the increasing concentration, the membrane hemolysis is decreased, and the membrane stabilization or protection is increased. Hence, anti-inflammatory activity of the extracts was concentration dependent.<sup>[16]</sup>

## CONCLUSION

Antioxidant and anti-inflammatory potentials of G-immune plus polyherbal formulation were evaluated. The formulation was found to possess antioxidant, radical scavenging activity, anti-inflammatory, protein denaturation inhibitor, and membrane stabilization effects, as determined by TAC, FRAP, CUPRAC, DPPH, ABTS, protein denaturation, and membrane stabilization assays. In general, results indicated that this polyherbal extract possesses potent bioactivities. Thus, it can be concluded that this polyherbal extract



**Figure 6:** Percentage inhibition of hemolysis by G-immune plus polyherbal formulation

formulation can be used as an antioxidant, anti-inflammatory, and immunomodulatory agent. Anti-inflammatory potential of the selected extract was depicted clearly in inhibition of albumin denaturation and membrane stabilization assay. Antioxidant and free radical scavenging potential is clearly revealed in TAC, FRAP assay, CUPRAC assay, DPPH radical scavenging assay, and ABTS radical scavenging assay. Thus, this G-immune plus capsule is a potent antioxidant and anti-inflammatory drug that can be used as an immunomodulatory agent boosting the human health.

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