



Research Article

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IN VITRO FREE RADICAL SCAVENGING POTENTIAL OF TRIMAD (A POLYHERBAL FORMULATION)

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Received on: 25/04/17 Accepted on: 12/06/17

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DOI: 10.7897/2277-4343.083183

ABSTRACT

The objective of the present study was to evaluate aqueous extract of *Trimad* for its *in vitro* antioxidant activity. A battery of *in vitro* biochemical tests such as total phenol content, ferric reducing power, ABTS [2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] scavenging activity and Lipid peroxidation inhibitory activity was used. The total phenol content of *Trimad* was found to be 279.5 mg/g dry mass. In case of DPPH scavenging activity, *Trimad* was found to be more potent than ascorbic acid at all given concentrations. It also showed significant ferric reducing potential (0.110% at 20µg/ml) and ABTS scavenging activity (31% at 80µg/ml). *Trimad* however showed very minimal (4.754% at 100µg/ml) lipid peroxidation activity. Our findings indicate promising antioxidant activity of *Trimad* extract however it needs further attention for its effective use in both modern and traditional system of medicines

Keywords: *Trimad*, Reducing power, Lipid peroxidation inhibitory activity, Free radical scavenging potential

INTRODUCTION

Trimad is a combination mentioned in Ayurveda that consists of three medicinal plants viz. *Cyperus rotundus*, *Embelia ribes*, and *Plumbago zeylanica*. All the three plants have been reported to possess a significant number of pharmacological and biological activities. *Cyperus rotundus* possesses anti-oxidant, anti-obesity, anti-candida, anti-inflammatory, anti-diabetic, anti-diarrhoeal, anti-mutagenic, anti-microbial, anti-bacterial, anti-pyretic, cytotoxic & apoptotic, cytoprotective as well as analgesic activities.¹⁻¹¹ *Embelia ribes*, besides being a good anti-oxidant, anti-bacterial and analgesic like *Cyperus rotundus*, has been shown to possess anti-anxiety, anti-cancer, anti-fertility activities with neuroprotective and cardioprotective action.¹², while *Plumbago zeylanica* is reported to be a potent anti-oxidant with anti-atherogenic, antifungal, antitumor, hepatoprotective, neuroprotective and cardioprotective activity.¹³ The phytochemical studies on these plants have revealed the presence of alkaloids, flavonoids, tannins, starch, glycosides, furochromones and many novel sesquiterpenoids.^{14,15, 16}

Although there is evidence available on antioxidant potential of individual ingredient of *Trimad*, the antioxidant activity as a whole (*Trimad*) has not been reported yet. Hence the present study was planned to evaluate the anti-oxidant potential of the aqueous extract of *Trimad* as a combination using various *in vitro* assays.

MATERIALS AND METHODS

Chemicals

DPPH (2, 2-diphenyl-1-picrylhydrazyl), gallic acid, ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonate) and Trolox were procured from Sigma-Aldrich, USA. Hydrochloric acid (HCl), potassium hexacyanoferrate {K₂Fe(CN)₆}, Methanol, Ferrous sulphate (FeSO₄), Ferric chloride (FeCl₃.6H₂O), and

Potassium dichromate (K₂Cr₂O₇) were procured from Qualigens Pvt. Ltd, Mumbai, India. Folin-Ciocalteu reagent and Thiobarbaturic acid (TBA) were purchased from Loba chemie, Mumbai, India. Potassium ferricyanide, Trichloroacetic acid, Butylated hydroxyl toluene (BHT), Potassium chloride (KCl), Ascorbic acid were purchased from Merck, Mumbai India. Copper sulphate (CuSO₄.5H₂O), Sodium potassium tartarate, Sodium carbonate (Na₂CO₃) And Sodium hydroxide (NaOH) was obtained from Fisher scientific, Mumbai, India.

Plant Extract preparation

Standardized aqueous extract of *Trimad* was procured from Pharmanza Herbals Pvt Ltd Gujrat.

Preparation of working standard and stock

One mg/ml, the extract was prepared and different dilutions were made from this stock solution.

Standards

Gallic acid was used as a standard for total phenol content. Ascorbic acid was used as a standard for DPPH-free radical scavenging activity, ferric reducing power assay and lipid peroxidation inhibitory activity. Trolox was used as a standard for ABTS scavenging activity.

All the assays were performed in triplicate and an average value was considered for calculation.

Total phenolic content

Total phenol content of *Trimad* was measured using equivalent of Gallic acid.¹⁷ Five ml of Folin-Ciocalteu reagent was added in 1 ml of sample then 4 ml of 1M sodium carbonate was added in

to it and incubated it in dark for 30 min at room temperature to measure the absorbance at 765 nm. The concentration range selected for this assay was 50, 100, 150, 200, 250 µg/ml.

A Gallic acid standard curve ($R^2 = 0.99$) was used to measure the phenolic content and expressed as mg/gm of dry mass of Gallic acid equivalents.

DPPH radical scavenging activity

In vitro DPPH-free radical scavenging activity of *Trimad* was determined by Brand-Williams *et al* method¹⁸. One ml of different concentrations (10 to 100 µg/ml) of *Trimad* extract was taken to which 5 ml of methanolic DPPH (33 mg/L) was added. The reaction mixture was incubated at 37°C for 30 min. Ascorbic acid was taken as control. The radical scavenging activity of the test samples was expressed as percentage inhibition and calculated according to the following formula

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

Where A_0 = Absorbance of the control, A_1 = Absorbance in the presence of samples. IC₅₀ values were calculated from the slope of the standard graph using 'y = mx + c' formula for every cases.

ABTS [2, 2 azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] scavenging activity

The method reported by Mukherjee *et al*¹⁹ and Dimitrova *et al*²⁰ was used to study ABTS activity.

ABTS radical cations (ABTS⁺) were produced by reacting ABTS solution (7 mM) with 2.45 mM potassium per sulphate in equal proportion. The mixture was allowed to stand in the dark at room temperature for 12-16 hours prior to use. The solution was then diluted by deionized water to obtain an absorbance of 0.7 units at 734nm. Fresh ABTS⁺ solution was prepared for each assay. 60µl extract was added to 2940µl of ABTS solution for 6 minutes in dark condition. The concentration range selected for this assay was 10- 100µg/ml. The Trolox (standard) curve was linear between 10µg/ml and 100µg/ml. The absorbance was read and percentage inhibition was calculated using formula:

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

Where,

A_0 = Absorbance of the control,
 A_1 = Absorbance in the presence of samples.

Ferric reducing power activity

Ferric reducing potential assay was performed as described by Oyaizu *et al*.²¹ 2.5 ml of phosphate buffer and potassium hexacyanoferrate was added in 1 ml of various concentrations of sample, then reaction mixture was incubated at 50°C for 15 min and then it was vortexed vigorously by adding 2.5 ml of trichloroacetic acid. 2.5 ml of upper layer was removed and mixed in 2.5 ml of distilled water and absorbance was measured at 700 nm by adding 0.5 ml of 0.1 % ferric chloride to the above reaction mixture. The concentration range selected for this assay was 20-100 µg/ml.

Lipid peroxidation inhibitory activity

The assay firstly involved perfusion of goat liver with 0.15 M KCl, then initiation of lipid peroxidation by addition of 1 mM FeCl₃, and this reaction was stopped by using or adding chilled 0.25 N HCl containing TCA and TBA (this starts the colour development). BHT was then added and finally absorbance was measured at 532 nm against solutions without FeCl₃ (normal) and without drug (induced) in double beam UV spectrophotometer. The concentration range selected for this assay was 100- 1000 µg/ml.

$$\% \text{ scavenging} = [(Absorbance \text{ of Induced} - Absorbance \text{ sample}) / (Absorbance \text{ Induced} - Absorbance \text{ Normal})] \times 100$$

IC₅₀ values were calculated from the slope of the standard graph using 'y = mx + c' formula for every cases.

RESULTS

Total phenolic content

Using this curve, the total phenol content in *Trimad* (10 µg/ml) was found to be 279.5 mg/g dry mass.

DPPH Radical scavenging activity

As expected, ascorbic acid which was used as standard demonstrated a dose dependent increase in DPPH radical scavenging activity. *Trimad* was found to be more potent than ascorbic acid at all given concentrations. This difference between the standard and test drug was statistically significant.

ABTS [2, 2 azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] scavenging activity

The standard Trolox showed a concentration dependent increase in ABTS scavenging activity. *Trimad* also followed similar pattern. At 80 µg/ml concentration, the activity of *Trimad* and standard was comparable (31%).

Ferric reducing power assay

The ferric reducing potential of *Trimad* was 0.110 (OD=700nm) at 20µg/ml which remained almost same at higher concentrations. Ascorbic acid showed a concentration dependent increase in the ferric reducing potential.

Lipid peroxidation inhibitory activity

The standard Ascorbic acid showed 92.05% inhibition of lipid peroxidation at very low concentration (10µg/ml). The lower concentrations of *Trimad* did not exhibit any significant activity. At 100µg/ml, the lipid peroxidation inhibitory activity of *Trimad* was 4.754% which then showed a concentration dependent increase with maximum inhibition of 34.607% at 1000 µg/ml.

DISCUSSION

In the present study, we evaluated anti-oxidant activity of aqueous extract of *Trimad* using a battery of tests. We observed that of the four assays used in the study, *Trimad* showed promising results in terms of DPPH and ABTS scavenging activity. In case of DPPH scavenging activity, the results were better even than the standard. *Trimad* extract however did not show much activity in terms of ferric reducing and lipid peroxidation inhibition.

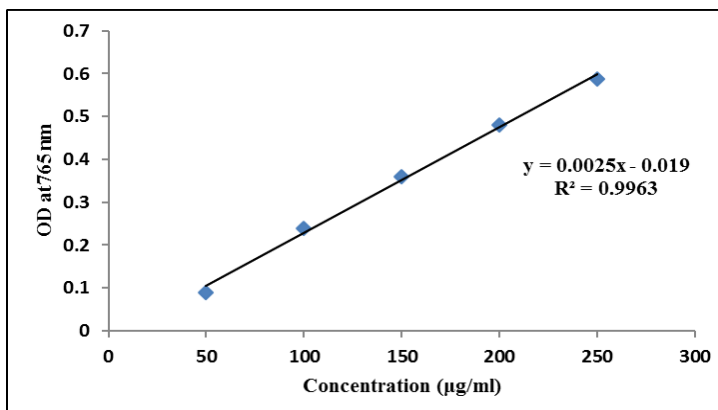


Figure 1: Total phenol content of standard (Gallic acid)

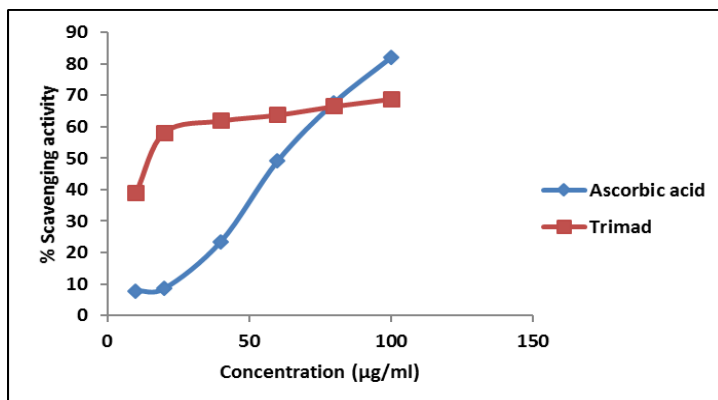


Figure 2: Percentage scavenging activity of DPPH free radicals by *Trimad* with Standard

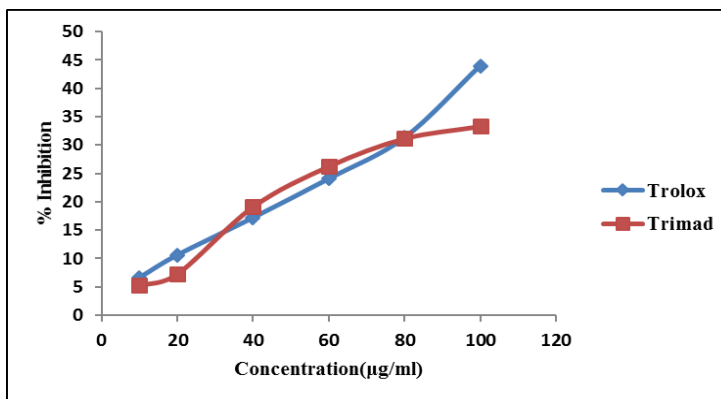


Figure 3: Percent inhibition of ABTS scavenging activity by *Trimad* with standard

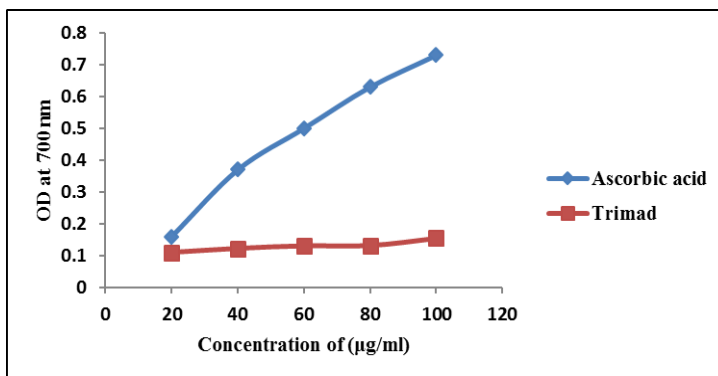


Figure 4: Ferric reducing potential of *Trimad* with standard

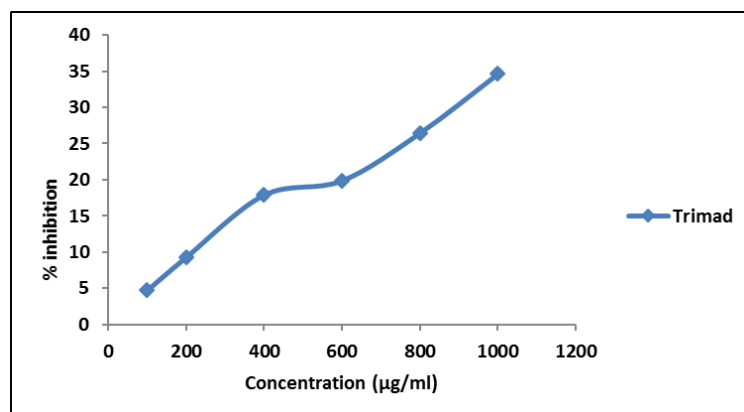


Figure 5: Percentage Lipid peroxidation inhibition of *Trimad*

Phenols are believed to be commonly known potent antioxidants.²² The total phenol content of *Trimad* was found to be 279.5 mg/g dry mass. In earlier studies, ethanolic extract of *E. ribes* has shown 31.9 g/kg of phenolic content²³; methanolic extract of *C. rotundus* has shown 27.40-37.85 mg GAE/g phenols²⁴ while methanolic extract of *P. zeylanica* is reported to have 109 g/kg phenolic content²⁵ The phenolic content of aqueous extract of *Trimad* was found higher as compared to phenol content of its ingredients. and the solvent (water) used for extraction.

Trimad has shown higher DPPH inhibition activity as compared to the standard Ascorbic acid at all concentrations. DPPH inhibition activity of individual ingredient of *Trimad* has also been reported. The aqueous extract of *C. rotundus* is reported to demonstrate a dose dependent increase in DPPH inhibition activity with maximum of 70% activity at 100 µg/ml concentration, while the ethanolic extract has shown dose dependent decrease with maximum of 70% activity at 10 µg/ml concentration.²⁶ The methanolic extract of *P. zeylanica* has reported to possess 73.41% activity at 100 µg/ml concentration, while the ethanolic extract has shown 50% activity at same concentration.²⁷ In our study, the *Trimad* aqueous extract was found to have around 70% activity at 100 µg/ml concentration. This was almost comparable with the reported activity of the different extracts of the individual ingredient.

In case of ABTS scavenging activity, *Trimad* has shown comparable activity with the standard Trolox at all studied concentrations. There are no reports available regarding ABTS scavenging activity of the individual ingredient of *Trimad*.

The ferric reducing potential of *Trimad* was comparable with the standard ascorbic acid only at lower concentration. While ascorbic acid showed dose dependent increase in the activity, the reducing capacity of *Trimad* remained almost same with increasing concentration. Although previous study has reported a direct relation between phenol content and reducing potential of plants.²⁸

Trimad showed lipid peroxidation inhibition activity at very high concentration as compared to standard indicating its minimal potential in this activity.

Thus, our study has demonstrated the differential anti-oxidant activity of *Trimad* in different assays. This activity can be further studied using different extracts and biological systems. These results suggest that the extract can prove effective in degenerative and life style diseases, where oxidative stress is a major pathological factor.

CONCLUSION

Our results reveal that the aqueous extract of *Trimad* shows DPPH radical scavenging activity and ABTS scavenging activity comparable to standard. The antioxidant activity of *Trimad* might be attributed to its polyphenolic content. The study suggests that *Trimad* is a natural source of antioxidants which may prove to be an effective therapeutic agent in preventing various oxidative stress related degenerative diseases. However, the components responsible for the anti-oxidative activity of aqueous extract of *Trimad* are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the herbal formulation. Detailed in vivo experiments may help to prove above results, which are in progress.

ACKNOWLEDGEMENT

We acknowledge Pharmanza Herbals, Gujrat Pvt Ltd. for providing us the aqueous extract of *Trimad* for the evaluation.

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Cite this article as:

Megha Salunke et al. *In vitro* free radical scavenging potential of Trimad (A polyherbal formulation). Int. J. Res. Ayurveda Pharm. 2017;8(Suppl 3):127-131 <http://dx.doi.org/10.7897/2277-4343.083183>

Source of support: Nil, Conflict of interest: None Declared

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