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EVALUATION OF ANTIOXIDANT ACTIVITY AND STANDARDIZATION OF ACT 12 AND ACT 13 FORMULATIONS

Dr. Shridhar Jitubhai Pandya* and Chetan Himmatbhai Savaliya

Director, R and D Department, Gplife Healthcare Pvt Ltd-708, Infinity Tower, Near Ayurvedic College, Lal Darwaja Rd, Surat, Gujarat 395003.

***Corresponding Author: Dr. Shridhar Jitubhai Pandya**
Director, R and D Department, Gplife Healthcare Pvt Ltd-708, Infinity Tower, Near Ayurvedic College, Lal Darwaja Rd, Surat, Gujarat 395003.

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ABSTRACT

Background and objective: COVID-19 is an infectious disease caused by the SARS-CoV-2 virus and spread over 211 countries worldwide and considered to be a pandemic. Globally on 28 August 2020, there are 24,299,923 confirmed subjects of COVID-19, including 827,730 deaths, and in India from Jan 30 2020 to 28 August 2020, there are 3,387,500 confirmed subjects and 61,529 deaths were reported. Covid-19 infection is due to the corona virus and leads to increased oxidative stress in the body, which leads to multiple-organ failure through various immune mechanisms. By considering the impact and intensity of the pandemic situation, Gplife Healthcare Pvt. Ltd. has come up with Act 12 and Act 13 formulations as the solution to reduce oxidative stress. **Materials and Methods:** Antioxidant activity of Act 12 and Act 13 was evaluated In-vitro by using DPPH scavenging assay, H2O2 radical scavenging assay, and Reducing power assay. Further, the standardization of both the formulations was performed by using the HPLC method. **Results:** The formulations Act 12 and Act 13 showed significant anti-oxidant action when compared with standard ascorbic acid. The percentage scavenging activity of Act 12 and Act 13 was found to be in the range of 18-53 % and 16-46 % respectively as compared to that of the standard at different concentrations. **Conclusion:** The study thus proves that Act 12 and Act 13 have significant antioxidant activities.

KEYWORDS: Oxidative stress; Antioxidant; COVID-19; Standardization; herbal formulation.

INTRODUCTION

It is commonly accepted that in a situation of oxidative stress reactive oxygen species (ROS) such as superoxide (O2-), hydroxyl (OH-), and peroxyl radicals are generated. The ROS plays an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation.[1]

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several examples have revealed the fact that plant which contains antioxidant potential demonstrate the beneficial effects in inflammatory diseases e.g. *Ledum groenlandicum* extracts possess antioxidant and anti-inflammatory activities, which supports it ethnopharmacological use.[2]

Considering the benefits of herbal components for their anti-oxidant activities Gplife Healthcare Pvt. Ltd. has developed Act 12 and Act 13 products. It comprises a synergistic combination of Phytoconstituents as ingredients reported to possesses immune-modulatory, anti-viral, immune-stimulant, anti-inflammatory, and anti-oxidant effects. ACT 12 is a tablet formulation that contains certain key ingredients out of a total of 12 ingredients are- Shilajit which is an immunomodulatory agent,[3] ginseng that possesses antioxidant, anti-inflammatory, and vasorelaxation activities,[4] Sea Buckthorn is an immunomodulatory agent,[5] Guduchi acts as an anti-stress and adaptogenic,[6] curcumin which is known for its antiviral, anti-inflammatory and antipyretic activity.[7] ACT 13 is a dry syrup formulation that consists of Liquorice- an antiviral and anti-HIV drug, Ajwain with its antiviral properties.[8] Tulsi which is known for its healing properties, anti-HIV, and antioxidant properties.[9] Ginger which possesses anticancer activity,[10] and Kakmachi which has anti-proliferative, antitumor, and immunomodulatory activity as major ingredients out of the total 13 ingredients. [11]

Taking into consideration the use of the above chemical constituents may prove beneficial in reducing oxidative stress. The present study was planned to investigate the antioxidant activity of the present study formulations. The standardization of the same was the aim of this research work.

MATERIAL AND METHODS

Materials

ACT 12 tablet formulation and ACT 13 dry syrup used for the analysis purpose aiming the evaluation of antioxidant activity and standardization of the same. HPLC-grade solvents such as methanol were obtained from Merck Ltd. Bangalore India. Standard was purchased from Sigma Aldrich.

Methods

A) Evaluation of antioxidant activity

a) DPPH radical scavenging activity

The ability of the formulations to scavenge DPPH radicals was determined by using the following method. 50 µl aliquot of each formulation, in 50 mm Tris-HCl buffer (pH 7.4), was mixed with 450 µl of Tris–HCl buffer and 1.0ml of 0.1mM DPPH in methanol. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated.

b) Hydroxyl radical scavenging activity

The degradation of Deoxyribose generated by the Fenton reaction was measured spectrophotometrically in the presence and absence of the test compound. The final reaction mixture in each test tube consisted of 0.3 ml each of Deoxyribose (30 mM), ferric chloride (1mM), EDTA (1 mM), H_2O_2 (20mM), in the phosphate buffer having pH 7.4 and 0.3 ml of test compound at different concentration. the test tubes were incubated for 30 min at 37^{o}C after incubation, trichloroacetic acid (0.5 ml, 5%) and the thiobarbituric acid (0.5 ml, 1%) were added and the reaction mixture was kept in a boiling water bath at 30 min. it was then cooled and the absorbance was measured at 532 nm. The result was expressed as a % of scavenging of hydroxyl radical.

c) Reducing power activity

The reducing power of the formulations was determined. Extracts at different concentrations in 1 ml of distilled water wee mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [K_3Fe (CN) 6] (1%), and then the mixture was incubated at 50^{0}C for 30 min. Afterward, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated an increase in reducing power.

2) Standardization by HPLC method

Preparation of Standard curcumin

5 mg of standard Curcumin was accurately weighed and transferred into a 20 mL volumetric flask. 15 mL of diluent was added and then sonicated in an ultrasonic water bath for 30 minutes. The solution was cooled and volume was made up to the mark with diluent. Then filtered through a 0.45 µ syringe filter. The resulting solution was used as a standard solution.

Preparation of the Test Solution

500 mg of ACT 12 was accurately weighed into a 100 mL volumetric flask. 70 mL of diluent was added and sonicated in an ultrasonic water bath for 30 minutes. The resulting solution was cooled and volume was made up to the mark with diluent. The content of the volumetric flask was filtered through Whatman filter paper No. 41 and then 0.45 µ syringe filter. The resulting solution was used as a test solution.

Chromatographic Conditions

HPLC was performed using a Shimadzu LC20AD system with a 2996 photodiode array detector (PDA). The standard compound was resolved on a reverse-phase 250 × 4.6 mm, 5-µm, Phenomenex C18 column 5µ (4.6 X 250 mm). The mobile phase was prepared from 0.1% ortho-phosphoric acid in the water of pH 2.5 (solvent-A) and Acetonitrile (100 v/v) (Solvent-B). The mobile phase was degassed and filtered through a 0.45-µm filter before use. The gradient program used is given in Table 1.

Table 1: The gradient program used for HPLC.

S. No.	Time	Flow	%A	%B
1	0	1.00	60.0	40.0
2	25.00	1.00	40.0	60.0
3	26.00	1.00	10.0	90.0
4	30.00	1.00	10.0	90.0
5	31.00	1.00	60.0	40.0
6	35.00	1.00	60.0	40.0

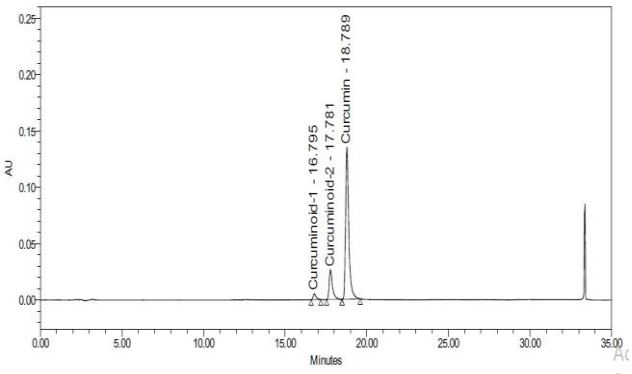


Figure1: The standardization chromatograph of Test compound along with a comparison with the standard curcumin obtained through HPLC.

The mobile phase flow rate was kept at 1 ml/min. Before the first injection, the column was saturated for 30 min with the initial mobile phase. The column temperature was maintained at 30°C. Injection volume was decided to maintain at 10 µl. The PDA was set by optimizing the wavelength at 425 nm to acquire the chromatogram. The standard compound was identified

by comparing the retention time and spectra obtained from the sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C.

Preparation of Calibration Graph

Seven different concentrations were prepared by diluting the standard stock solution. The calibration graph of each standard was constructed by plotting concentrations against peak area for the respective standards.

Validation of HPLC Method

The proposed HPLC method was validated in terms of specificity, precision, accuracy, standard solution stability, sample solution stability, and robustness as per the International Conference on Harmonization (ICH) guidelines.[14-15]

1. Specificity

The specificity of the method was studied by assessment of peak purity of Standard using the Waters empower software and diode array detector and represented in terms of purity angle, purity threshold, and purity flag.

2. Precision

Precision was studied in terms of system precision, methodprecision, and intermediate precision.

3. System Precision

System precision was carried out by six replicate injections from the same vial of standard and was expressed in terms of percent relative standard deviation (% RSD) tailing, plate count, and resolution.

4. Method Precision

The six separately prepared samples were analyzed by the proposed procedure. The % assay for each analyte was expressed in terms of % RSD.

5. Intermediate Precision

Intermediate precision was performed on different systems, one on Waters e2695 Alliance system with a 2996 PDA and the other on 2489 ultraviolet (UV) detector by different analysts by analyzing six different samples of extract and was expressed in terms of % RSD.

6. Recovery Studies

The accuracy of the method was determined from recovery studies by adding a known amount of standard at the 80%, 100%, and 120% level to the pre-analyzed sample followed by replicate quantitative analyses by the proposed method.

7. Solution Stability

The standard and sample solutions were prepared as per the proposed method and subjected to stability study at 25°C for 24 h. The standard and test solution was analyzed at initial and at different time intervals of 4 h up to 24 h. Change in the response of compound in the test solution with respect to time was calculated as absolute percent difference against initial response.

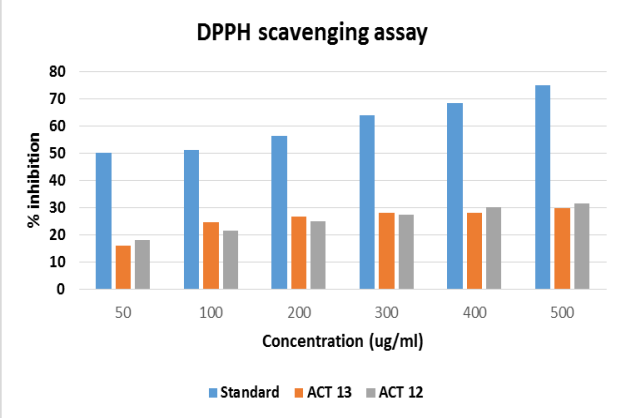
8. Robustness

The robustness of the method was determined by a small deviation in the method parameters. The parameters selected were deviation in wavelength, column temperature, and flow rate. The retention time of the test compound and % RSD was determined using system suitability parameters.

RESULTS

DPPH radical scavenging activity

All the concentrations were capable of scavenging DPPH radicals at pH 7.4 in a dose-dependent fashion. All the concentrations of the test compound were capable of scavenging the free radicals and had shown anti-oxidant activity. Both the formulations showed equivalent activity. Graph 1 depicts the percentage inhibition of ACT 12 and ACT 13 at different concentrations of test compounds and also the comparison with different concentrations of Standard i.e. ascorbic acid. The % inhibition of ACT 12 formulation at concentrations of 50 ug/ml, 100 ug/ml, 200 ug/ml, 300 ug/ml, 400 ug/ml, 500 ug/ml were found to be 18.22 %, 21.67 %, 25.12 %, 27.58 %, 30.29 %, 31.52% respectively. Whereas the % inhibition of ACT 12 formulation at concentrations of 50 ug/ml, 100 ug/ml, 200 ug/ml, 300 ug/ml, 400 ug/ml, 500 ug/ml were found to be 16.00 %, 24.63 %, 26.84 %, 28.07 %, 28.32 %, 30% respectively. When compared statistically between the groups, the P-value was found to be 0.0013 and the test results were found to be significant.

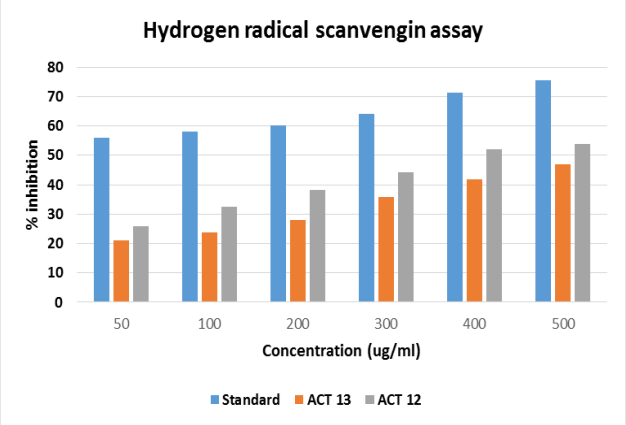


Graph 1: The graphs show % inhibition of ACT 12 and ACT 13 as compared to standard at different concentrations of test compound and standard.

Hydrogen radical scavenging activity

Hydroxyl radicals are very reactive, can be generated in biological cells to the Fenton reaction. The test compounds exhibited concentration-dependent scavenging activity against hydroxyl radical generated in the Fenton reaction system. ACT 12 and ACT 13 formulations demonstrated scavenging of hydroxyl radicals rangingfrom 21.17 % to 53.76 %. The P-value

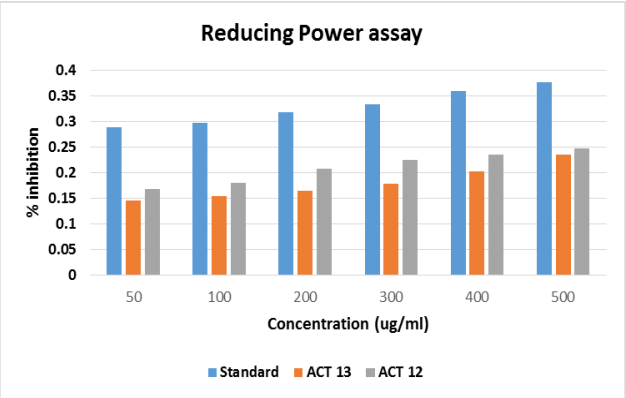
was calculated and the results were found to be significant. The results are depicted in Graph 2



Graph 2: It indicates the % inhibition of ACT 12 and ACT 13 in comparison with Standard.

Reducing Power Assay

For the measurements of the reductive ability of test compounds, the Fe^{3+} – Fe^{2+} transformation was investigated in the presence of test compounds. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power of test compounds enhanced with increasing concentration of samples. The absorbance of the samples increased together with the reducing power. The reducingpower of test compounds was found to be concentration. The results are depicted in Graph 3.



Graph 3: The above graph depicts the absorbance of ACT 13 and ACT 12 in comparison with the standard.

DISCUSSION

Oxidative stress is the result of an imbalance in the body between the oxidizing system, consisting mainly of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS),[12] and antioxidant systems. SARS-CoV2, probably like other RNA viruses can trigger oxidative stress. A possible oxidative storm with all the deleterious effects of RONS, notably lipid peroxidation and proteins oxidation of membranes which can contribute to the transformation, hyalinization of pulmonary alveolar membranes with lethal respiratory distress.[13] It is triggered by a wide variety of viral infections[14] including HIV 1, viral hepatitis B, C, D viruses, herpes viruses, respiratory viruses, most of the RNA viruses[15] probably also coronaviruses belonging to this family. the classic coronaviruses, responsible for moderate respiratory infections in general, the SARS-CoV and MERS-CoV involved in epidemics of more severe respiratory infections,[16] and the new coronavirus (SARS-CoV2) discovered in January 2020 responsible for an infectious disease called COVID-19 which is currently experiencing a worldwide outbreak.[17] Generally, viral infections lead to an increase in the production of free radicals and a depletion of antioxidants. The mode of action varies according to the viruses as demonstrated by the analysis of the oxidative stress induced by different viruses of the Flaviviridae family[14] but we find these two phenomena increasing the oxidative stress in these RNA viruses' infections and for Ivanov[15] one of the sources of production of these ROS could be the mitochondrial dysfunction caused by the penetration of the virus into the cell known as cytokine storm"

Cytokine storm (Overactivation of the immune response) and poor prognosis in COVID-19 is the most evident fact in clinical management. It has long been believed that cytokines play an important role in immunopathology during viral infection. A rapid and well-coordinated innate immune response is the first line of defense against viral infection. However, dysregulated and excessiveimmune responses may cause immune damage to the human body. Increased levels of cytokines are indicative of severe infections and include IL-2, IL-2R, IL-6, IL-7, IL-8 IL-10, IP10, MIP1A, and TNF-α.[18] High levels of plasmatic IL-6 have been consistently reported in COVID- 19 and even appear to be associated with poor prognosis and risk of death.[19] There is consensus that in severe COVID-19 infection, an exacerbated pulmonary and systemic inflammatory response occurs, with increased serum levels of inflammatory markers, such as C-reactive protein (CRP) and lactic dehydrogenase (LDH) all of which may result in cytokine storm similar to SARS and MERS.[20] The serum levels of cytokines are significantly increased and the degree of increase is positively correlated with the mortality rate. The cytokine storm is also a key factor in determining the clinical course of extrapulmonary multiple-organ failure.

Considering the impact of oxidative stress and the intensity of impact on the population at the times of this pandemic situation. GP life Healthcare Pvt. Ltd. has developed Act 12 and Act 13 formulations as a solution

to reduce this oxidative stress. The current study aims to evaluate the antioxidant activity of Act 12 and Act 13 formulations. Both the formulations were compared with standard ascorbic acid for their anti-oxidant activity and the results were found to be significant. To confirm the presence of activity was evaluated by performing three anti-oxidant assays namely: DPPH scavenging assay, Hydrogen radical scavenging assay, and reducing power assay. Further standardization of both the formulations was performed by the HPLC method.

CONCLUSION

The ACT 12 and ACT 13 formulation which is a tablet formulation and a dry syrup formulation was tested for its antioxidant activity by Invitro analysis, and standardization of the both was carried out by using HPLC method. From the results it can be concluded that both the formulations shows potent anti-oxidant activity. Thus, the formulation can have widescale applications in treating various diseases due to oxidative stress. Considereing the current pandemic situation, the presented formulation may be useful in treating the Covid 19 infection by reducing the oxidative stress caused due to the viral infection. From the above study, both the fomulations had shown potential antioxidant activity.

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