IN VITRO ANALYSIS OF ANTIOXIDANT ACTIVITY IN POLYHERBAL PLANT EXTRACT

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Abstract

Most plants are considered to prevent free radicals associated damages by numerous ways including direct scavenging of free radicals and inhibition of enzymes involved in free radical production. The aim of the present study was to examine the antioxidant activities of the hexane from polyherbal plants of Carica papaya, Punica granatum, Catharanthus roseus and Cympbopogan citratus which is long been known to be a very important source of pharmaceutical. Different concentrations of polyherbal extract was tested for anti-oxidant properties using hydrogen peroxide assay. Similarly, the free radical scavenging activity was determined by DPPH assay. The results showed that the polyherbal extract showed a strong inhibition value at IC_{50} of concentration of 170.7 µg/ml, 229.57 µg/ml. This study provides evidence that the polyherbal formulation possesses a strong antioxidant activity. Therefore, it might be beneficial as medicinal plant as an antioxidant and anticancer nutraceutical and pharmaceutical sources.

Keywords: *Antioxidant activity, DPPH assay, Hexane, Hydrogen peroxide assay*

1.Introduction

Skin acts as a protective barrier for the body which protects the body from the external environment. Epidermal layer of skin can be damaged due to the wound [1]. Which is defined as the disruption of the integrity of the skin by various factors such as pressure, trauma, animal or insect bites, and mechanical abrasions. Invasion of various pathogenic microorganisms at the wounded tissue

results in severe chronic wound infection. Anatomical and functional integrity of tissue gets disrupted due to this chronic wound infection. Worldwide, it is estimated that at least 6 million people suffer from chronic wound infections every year [2]. Wound healing is natural phenomenon by which body restores the cellular and functional continuity of tissue [3].Appropriate wound healing is necessary to regain the functional and anatomical status of the damaged tissue that got disturbed due to wound. Various complex biochemical events are involved in appropriate healing of wound. These events are divided into three phases: Inflammatory phase, proliferative phase, and remodeling phase [4].

Wound infection with the pathogenic microorganisms such as *Staphylococcus aureus, Escherichia coli,* and *P. aeruginosa* results in delay of physiological events involved in the healing of wound [5]. Antioxidants also act as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, and metal chelating agents. Due to the effect on immune system, there is a need for natural antioxidants (safe and nontoxic) as compared to synthetic antioxidants (toxic for human) [6].

Plants contain many constituents with local physical impact on body tissues, and the topical use of herbal remedies is among the most noticeable in the simplest traditions of health care [7]. To support the usage of selected plant extracts in Ayurveda, the antioxidant potential of the fruits of *Momordica charantia* Linn., bark of *Eugenia jambolana* Linn., fruits of *Ziziphus mauritiana* Lam., and bark of *Acacia catechu* Willd. of Indian origin was examined.

Carica papaya, belongs to the family of Caricaceae, and several species of Caricaceae have been used as remedy against a variety of diseases [8]. Originally derived from the southern part of Mexico, *Carica papaya*is a perennial plant, and it is presently distributed over the whole tropical area. In particular, *Carica papaya*fruit circulates widely, and it is accepted as food or as a quasi drug. Lot of scientific investigations have been conducted to evaluate the biological activities of different parts of *Carica papaya*, including fruits, shoots, leaves, rinds, seeds, roots or latex. The leaves of papaya have been shown to contain many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, cystatin, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates [9].

The objective of this work was to assess the antioxidant activity of the combination of extract (polyherbal formulation [PHF]) by *in vitro* studies and relate them with ascorbic acid, a known antioxidant.

2. Materials and Methods

2.1. Collection of plant material

Carica papaya, Cymbopogon citratus, Punica granatum L., Cantharanthus roseus the plant species were collected from the wild source which was available in Trichy District, Tamil Nadu, India.

2.2. Hydrogen peroxide scavenging assay

2.2.1. Principle

Hydrogen peroxide is a weak oxidizing agent and it can inactivate some enzymes directly by oxidation of essential thiol groups (-SH). H_2O_2 can cross cell membranes rapidly, once it goes inside the cell, mostly react with $Fe^{2+,}$ and possibly Cu^{2+} ions to form hydroxyl radical. This may be the origin of many of its toxic effects. It is therefore biologically beneficial for cells to control the amount of H_2O_2 that is allowed to accumulate.

2.2.2. Materials Required

Hydrogen peroxide solution and Sodium phosphate buffer.

2.2.3. Procedure

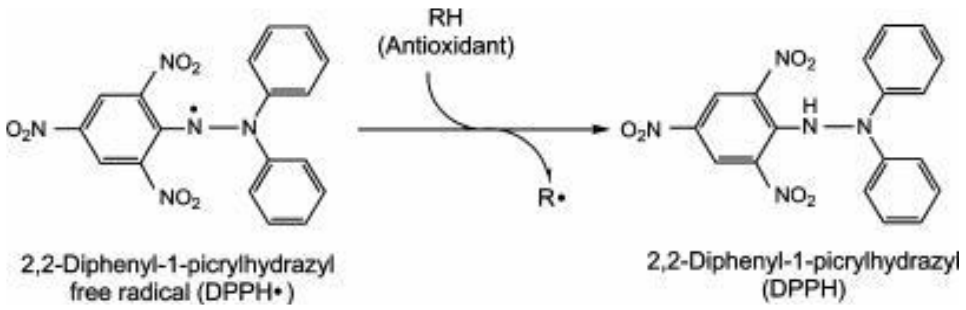
Ability of plant extracts to scavenge hydrogen peroxide was estimated according to the method reported by Ruch *et al*. with minor modification. A solution of H_2O_2 (43 mM) is prepared in phosphate buffer (1 M pH 7.4). Different concentration of polyherbal extract sample (500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml and 5 µg/ml) was added to H_2O_2 solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. The free radical scavenging activity was determined by evaluating % inhibition as above.

% inhibition = [(Control- Test)/control] ×100.

2.3. DPPH Radical scavenging activity

2.3.1. Principle

The DPPH assay is popular in natural product antioxidant studies because it is very simple and sensitive. DPPH assay is based on the concept that a hydrogen donor is an antioxidant. This assay measures compounds which are radical scavengers. Figure 1, below, shows the mechanism by which DPPH accepts hydrogen from an antioxidant. DPPH is one of the few stable as well as commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples. Monitoring DPPH with a UV spectrometer has become the most commonly used method because it is very simple and accurate. DPPH shows a strong absorption maximum at 517 nm (purple). The color changes from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This is a stoichiometric reaction with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.



2.3.2. Materials Required

0.1mM DPPH solution, Ascorbic acid, Methanol

0.1 mM DPPH Solution

Dissolve 39 mg of DPPH in 100 ml of methanol and store at -20^o C until needed.

Ascorbic acid (Standard)

1mg/ ml of Ascorbic acid

2.3.3. Procedure

1. Briefly, prepare 0.1 mM of DPPH solution in methanol and add 100 µl of this solution to 300 µl of the solution of Poly herbal formulation sample at different concentration (500, 250, 100, 50 and 5 µg/mL).
2. The mixtures have to be shaken vigorously and allowed to stand at room temperature for 30 minutes.
3. Then the absorbance has to be measured at 517 nm using a UV-VIS spectrophotometer. (Ascorbic acid can be used as the reference).

4. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.
5. The capability of scavenging the DPPH radical can be calculated by using the following formula.
6. DPPH scavenging effect (% inhibition) = [(absorbance of control - absorbance of reaction mixture)/absorbance of control] X 100

2.4. Phytochemical analysis

2.4.1. Detection of resins

To 0.5 of plant extract, 3ml of $CuSo_4$ solution is added. Shake for about 1-2 min formation of green colour ppt indicates the presence of resins.

2.4.2. Detection of Carboxylic acid

To 1ml plant extract, 2ml of sodium bicarbonate solution is added. Colour changes occur indicates the presence of carboxylic acid.

2.4.3. Detection of Tannins

To 2ml of plant extract, 2-3ml of 10% HCL is added and boiled for 5-6 min. Formation of red colour indicates the presence of tannins.

2.4.4. Detection of Steroids

To 0.5 ml extract, 5ml of chloroform is added and equal amount of conc. H_2SO_4 is added. In the upper layer formation of red colour is and in the lower layer, yellow with green colour is formation indicates the presence of steroids.

2.4.5. Detection of Flavanoids

To 0.5 ml extract, add 4ml of 1% ammonia and to this add 1ml of conc. H_2SO_4. Formation of yellow colour indicates the presence of flavonoids.

2.4.6. Detection of Carbohydrates

To 0.5ml of extract, 0.5ml of Benedict reagent is added ad boiled for 2 min. Colour changes and precipitate is formed. It indicates the presence of carbohydrate.

2.4.7. Detection of Glycosides

To prepare Hydrosalyte :To 50mg of extract, 2ml of conc. HCL is added and kept in water bath for 1 hour and then filteration methods. The filtrate is hydrated.

2.4.8. Born- Trageru's Test

Take 2ml of hydrolysate, add 3ml of chloroform, shake vigorously, then the chloroform layer gets separated. To formation 10% ammonia solution of pink colour indicates the presence of glycosides.

2.4.9. Saponification test

To 1 or 2ml of normal sodium hydroxide, 2mlof extract is added and boiled for 2 minutes. Formation of soap or fat indicates the positive test for saponification.

2.4.10. Detection of Proteins

Estimation of Brad Ford Method: To 500 and 1 of extract, add 5mlof brad ford reagent, Take

OD at 575nm.

2.4.11. Detection of Phenol

Ferric Chloride Test : To 50 mg of extract, 5ml distilled water, few drops of 5% ferric chloride solution, dark green colour. Indicates the presence of phenol.

2.4.12. Biuret Test :

To 2ml of extract, 1 drop of 2% $CuSo_4$ solution. Add 1 ml of 95% ethanol, then add 2 to 3 sodium hydroxide pellets.Formation of pink colour indicates the test is positive.

2.4.13. Sapon Test :

To 50 mg of extract,20 ml of distilled water. Shake vigorously for 15 min, at 2 cm layer of foam formation indicates the presence of saponins.

2.4.14. Gum Test:

To 100 mg extract. Dissolved in 2 ml of distilled water. 2ml of absolute alcohol with constant stirring. White colour cloudy ppt indicates gums & mucilage's.

2.4.15. Detection of Flavanoglycoside:

50 mg extract is dissolved in 5ml ethanol. Add few drops of magnesium sulphate & few drops of conc.HCL Formation of pink colour. Presence of Flavanoglycoside.

3. Results and Discussion

3.1. H_2O_2 assay

In recent years much attention has been devoted to natural antioxidants and their health benefits. Antioxidant-based drug formulations are used for the prevention and treatment of many complex diseases. Plants are a major source of natural antioxidants; they produce a wide range of secondary metabolites with antioxidative activities that have therapeutic potential. Polyphenols are the most abundant antioxidant compounds of plant raw material. Their antioxidant activity is based on to their redox properties, which facilitate their activity as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin. The reducing ability is generally associated with the presence of reductants which exert antioxidant action through breaking the free radical chain by donating a hydrogen atom or preventing peroxide formation. In the present study, the polyherbal extract showed excellent antioxidant activity by H_2O_2 assay. The IC50 concentration of polyherbal extract was found to be µg/ml. The polyherbal formulation inhibited 46.63 % of free radicles at the concentration of 500 µg/ml.

3. 1. 1. OD Value at 230 nm

Control Mean OD value: 1.681

TABLE 1: OD values of tested sample concentration at 230nm in triplicates forH_2O_2 assay

S. No	Tested sample concentration (µg/ml)	OD Value at 230 nm (in triplicates)		
1.	Control	1.705	1.648	1.691
2.	500 µg/ml	0.779	0.940	0.972
3.	250 µg/ml	0.552	0.598	0.611
4.	100 µg/ml	0.114	0.140	0.119
5.	50 µg/ml	0.122	0.117	0.101
6.	5 µg/ml	0.094	0.109	0.105

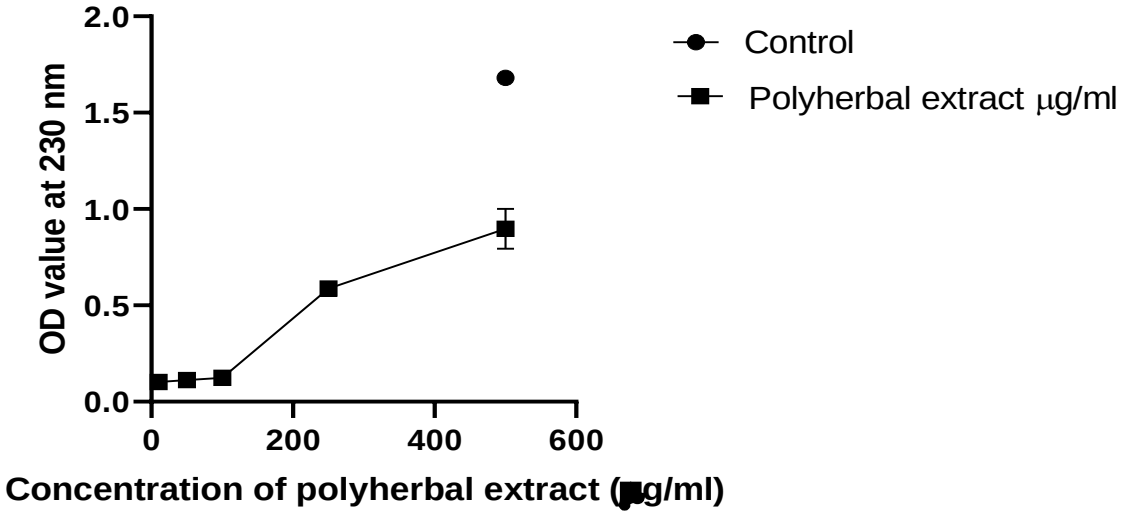


FIGURE 1: OD value graph of tested sample at various concentrations at 230nm in triplicates for H_2O_2 assay

3.1.2 Percentage of inhibition

TABLE 2: Percentage of inhibition in the tested sample at given concentrations for H_2O_2 assay

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value
1.	Control	100	100	100	100
2.	500 µg/ml	53.65	44.08	42.17	46.63
3.	250 µg/ml	67.16	64.42	63.65	65.07
4.	100 µg/ml	93.21	91.67	92.92	92.6
5.	50 µg/ml	92.74	93.03	93.99	93.25
6.	10 µg/ml	94.40	93.51	93.75	93.88

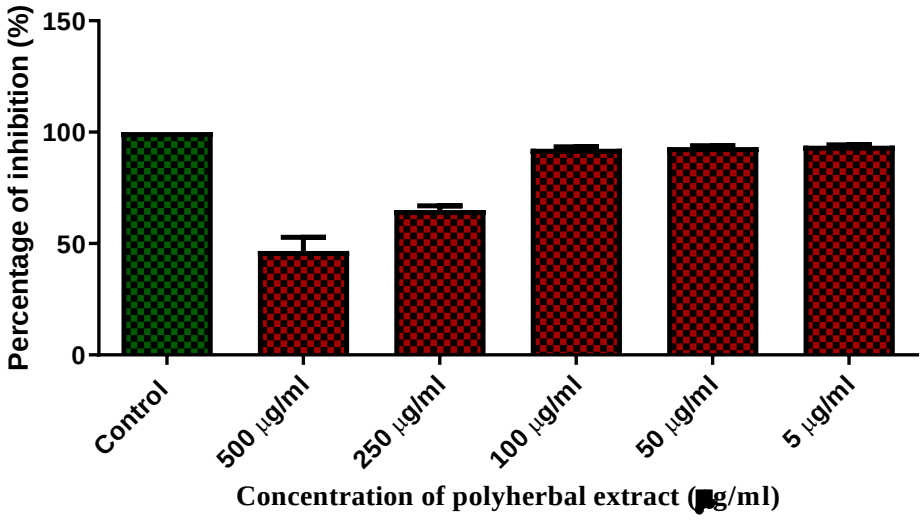


FIGURE 2: Percentage graph of inhibition in the tested sample at given concentrations

3.1.3 IC50 Value of tested sample: 229.5 µg/ml

TABLE 3: IC50 Value tabulation for the H_2O_2 assay

log(inhibitor) vs. normalized response -- Variable slope	Polyherbal extract µg/ml
Best-fit values	
LogIC50	2.361
HillSlope	5.295
IC50	229.5
Std. Error	
LogIC50	0.01837
HillSlope	2.073
95% CI (asymptotic)	
LogIC50	2.321 to 2.400
HillSlope	0.8166 to 9.772
IC50	209.4 to 251.4
Goodness of Fit	
Degrees of Freedom	13
R squared	0.9841
Sum of Squares	401.1
Sy.x	5.554
Number of points	
# of X values	15

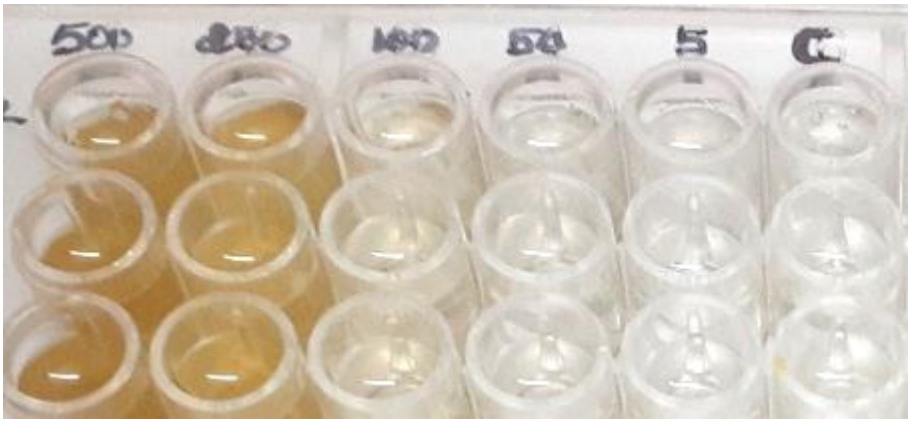


FIGURE 3: H_2O_2 assay for the polyherbal extract

3.2. DPPH Radical scavenging activity

Free radicals which are delivered as a consequence of typical biochemical responses in the body are involved in cancer, ischemic heart disease, inflammation, diabetes, aging, atherosclerosis, immunosuppression, and neurodegenerative disorders. The human body has characteristic barrier system to counter free radical as proteins, for example, catalase, superoxide dismutase, and glutathione peroxidase. Selenium, vitamin C, carotene, vitamin E, lycopene, lutein, and different carotenoids have been utilized as supplementary antioxidants. Hence, the secondary metabolites of the plant as flavonoids and terpenoids act an important role in the defense against free radicals. Here in this study, the polyherbal extract exhibited an potential anti-oxidant activity by DPPH assay at the IC50 concentration.

3.2.1. OD Value at 517 nm

Control Mean OD value: **3.005**

TABLE 4: OD values at 517nm for tested sample at various concentrations for DPPH assay

S. No	Tested sample concentration (µg/ml)	OD Value at 517 nm (in triplicates)		
1.	Control	2.970	3.168	2.879
2.	500 µg/ml	0.970	0.870	0.888
3.	250 µg/ml	0.807	0.674	0.797
4.	100 µg/ml	0.659	0.746	0.707
5.	50 µg/ml	0.666	0.625	0.653
6.	5 µg/ml	0.583	0.606	0.612
7.	Ascorbic acid	0.08	0.11	0.12

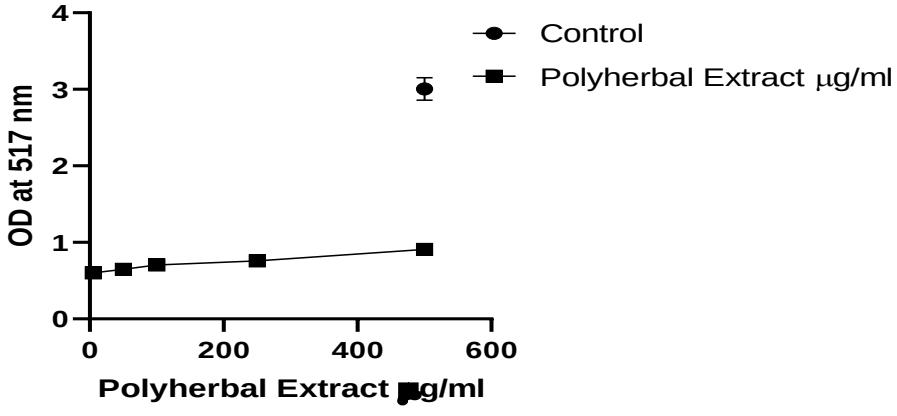


FIGURE 4: OD values graph at 517nm for tested sample at various concentrations for DPPH assay

3.2.2. Percentage of inhibition

TABLE 5: Percentage of inhibition in the tested sample at given concentration for DPPH assay

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
7.	Control	100	100	100	100
8.	500 µg/ml	67.72	71.04	70.44	69.73
9.	250 µg/ml	73.14	77.57	73.47	74.72
10.	50 µg/ml	78.06	75.17	76.47	76.56
11.	10 µg/ml	77.83	79.20	78.26	78.43
12.	5 µg/ml	80.59	79.83	79.63	80.01
13. a	Ascorbic acid	97.33	96.33	96	96.55

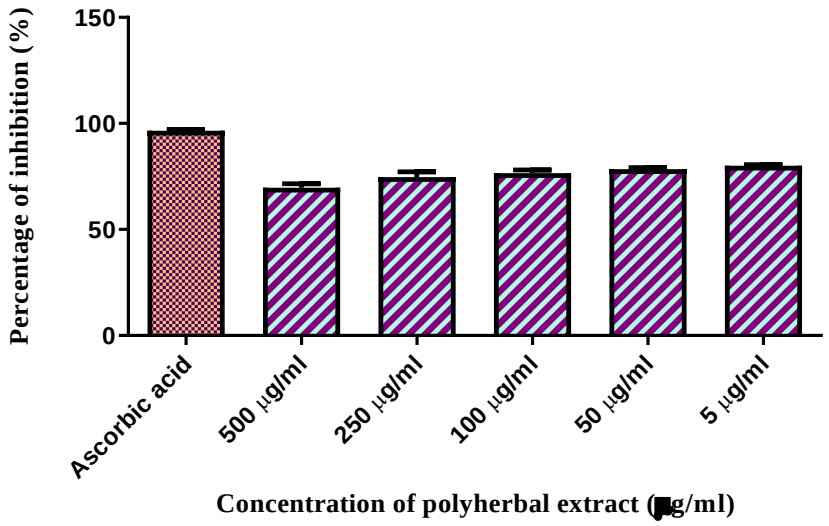


FIGURE 5: Percentage graph of inhibition in the tested sample at given concentrations for DPPH assay

3.2.3 IC50 Value of tested sample: 170.7 µg/ml

TABLE 6: IC50 Value tabulation for the DPPH Assay

log(inhibitor) vs. normalized response -- Variable slope	Polyherbal Extract µg/ml
Best-fit values	
LogIC50	2.232
HillSlope	1.618
IC50	170.7
Std. Error	
LogIC50	0.07344
HillSlope	0.4058
95% CI (asymptotic)	
LogIC50	2.074 to 2.391
HillSlope	0.7409 to 2.494
IC50	118.5 to 246.0
Goodness of Fit	
Degrees of Freedom	13
R squared	0.8228
Sum of Squares	3581
Sy.x	16.60
Number of points	
# of X values	15

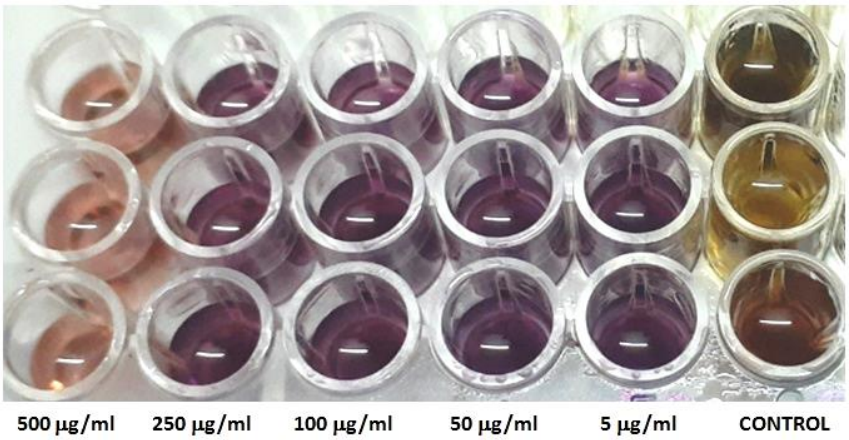


FIGURE 6: DPPH Assay for the polyherbal extract

3.3. Phytochemical analysis

Phytochemical characterization of polyherbal formulation was carried out using qualitative assay method. The results showed the presence of resins, carboxylic acid, steroids, carbohydrates, glycosides and phenol compounds.

TABLE 7: Results tabulation of the phytochemical analysis

S. No	Test	Result
1	Resins	Present
2	Carboxylic Acid	Present
3	Tannins	Absent
4	Steroids	Present
5	Flavonoids	Present
6	Carbohydrates	Present
7	Glycosides	Present
8	Saponification	Absent
9	Protein(OD Value)	0.64
10	Phenol	Present
11	Biuret	Absent
12	Saponin	Absent
13	Gum test	Absent
14	Flavanoglycoside	Absent
15	Alkaloids	Absent

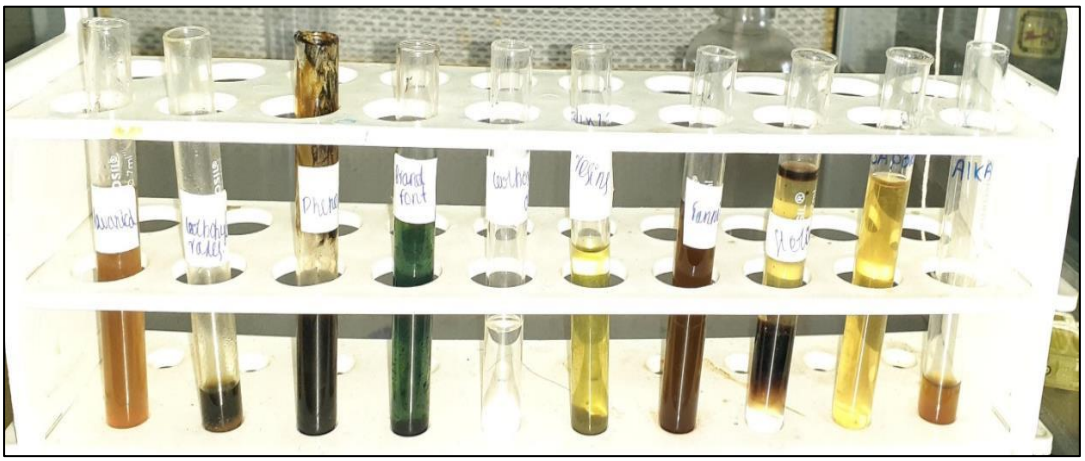


FIGURE 7

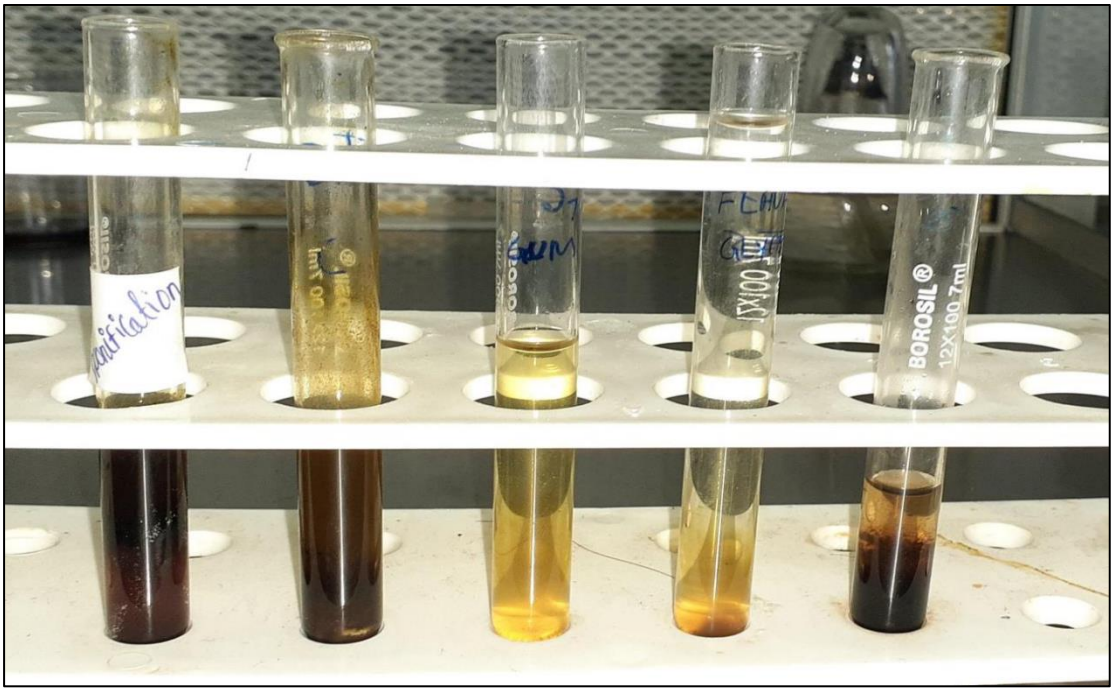


FIGURE 8

Punica granatum L.

Carica papaya L.

Cantharanthus roseus L.

Cymbopogon citrates L.

Polyherbal Plant extraction

FIGURE 9: Plant extracts and collected polyherbal mixture

4. Conclusion

The results from this study indicate that they possess antioxidant properties and could serve as free radical inhibitors, acting possibly as primary antioxidants. Since reactive oxygen species are thought to be associated with the pathogenesis of AIDS, and HIV-infected individuals often have impaired antioxidant defenses, the inhibitory effect of the extracts on free radicals may partially justify the traditional use of these plants in the management of OFIs in HIV patients in South Africa.

5. Acknowledgments

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6. References

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