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EVALUATION OF IN VITRO ANTIOXIDANT CAPACITY AND REDUCING POTENTIAL OF POLYHERBAL FORMULATION OXIWELL: A NUTRITIONAL SUPPLEMENT

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ABSTRACT

In biological system, reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide, hydroxyl, and nitric oxide radicals, can damage the DNA and lead to the oxidation of lipid and proteins in cells. Normally, antioxidant system occurring in human body can scavenge these radicals, which would keep the balance between oxidation and anti-oxidation. Nonetheless, the exposure of cigarette smoking, alcohol, radiation, or environmental toxins induces the production of excessive ROS and RNS, which disrupt the balance between oxidation and anti-oxidation and result in some chronic and degenerative diseases. Many herbs contain antioxidant compounds which protects the cells against the damaging effects of reactive oxygen species. Polyherbal formulations containing different herbs are used for the treatment of various diseases. The study revealed a very good in vitro free-radical scavenging properties of oxiwell a polyherbal formulation from ayurwell labs which contains the extracts of *Mangifera indica*, *Withania somnifera*, *Glycyrrhiza glabra*, *Vitis vinifera*, powders of *Emblica officinalis*; and oils of *Triticum sativum*. The antioxidant activity of this formulation was evaluated by measuring reducing ability, free radical scavenging activity by DPPH and hydrogen peroxide methods.

KEYWORDS: Free radicals, antioxidants, polyherbals.

INTRODUCTION

Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to

treat various human ailments because they contain the components of therapeutic value. In addition, plant based drugs remain an important source of therapeutic agents because of the availability, relatively cheaper cost and non-toxic nature when compared to modern medicine.[1] Many herbs contain antioxidant compounds which protects the cells against the damaging effects of reactive oxygen species. Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide play a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and parkinson's disease. The free radicals in the human body are generated through aerobic respiration or from exogenous sources.[2,3]

Free radicals produced in the body react with various biological molecules namely lipids, proteins and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants. Even though our body is safeguarded by natural antioxidant defense, there is always a demand for antioxidants from natural sources.[4]

Role of antioxidant based drugs in biological system.

Today, herbal medicines are manufactured similarly as the different dosage forms of the modern system. Though the plants have been proven for their efficacy since centuries, the documentation of the evidences are not available for every practice. Hence, herbal medicines need to be revalidated at present for the evidence based medicine practice.[5,6]

In the advent of the internationally accepted protocol for the evaluation of a drug and with due consideration to the established understanding of the plants, as is available in the literature of Ayurveda, a better utilization may be achieved.[7]

What are Free radicals

Free radicals are highly reactive substances formed in the body's cells as a result of metabolic processes.[7] In 2001 Gerschman *et al.*, first proposed the theory of free radical formation. A free radical is a molecule with one or more unpaired electrons in its outer orbital. Many of these molecular species are oxygen (and sometimes nitrogen) centered. Oxygen free radicals and its non-radical products are associated with reactive oxygen species.[8,10]

What are Antioxidants?

Antioxidants are substances that scavenge free radicals and help to decrease the incidence of oxidative stress induced damage.[9] Recent developments in the field of medicine reports a number of diseases associated with free radicals. The risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure to chemicals, pollution, smoking, drugs, illness, stress, etc.

MATERIAL AND METHODS

MATERIALS

Chemical compounds

1, 1-Diphenyl–2-picrylhydrazyl (DPPH), Nitro blue tetrazolium (NBT), Ethylene diamine tetra acetic acid (EDTA), dimethyl sulphoxide (DMSO), Riboflavin. Trichloroacetic acid, nitric oxide, sulphanilamide, chromophore, ammonium molybdate, citric acid monohydrate, potassium ferricyanide, trichloroacetic acid.

Apparatus and equipment's.

General laboratory glassware including volumetric flask, measuring cylinder, beakers, pestle and mortar, filter papers, test tubes, micro pipette, weighing balances and water bath. Specific equipment used in the study included centrifuge apparatus (REMI-2000), incubator, and a spectrophotometer (Jasco V-630) was also used in the present research work.

Herbal Formulation

Oxiwell, a phytopharmaceutical formulation of the Ayurwell labs was purchased from ShivShakti medical store, Dawa bazaar, Lashkar, Gwalior (M.P.) for the present research work. Oxiwell is recommended in the management of oxidative stress associated with Coronary artery disease, Diabetes mellitus, Oral submucous fibrosis and other conditions by inhibiting oxidation reactions, thereby preventing the damage caused by free radicals. Oxiwell is containing the extracts of *Mangifera indica*, *Withania somnifera*, *Glycyrrhiza glabra*, *Vitis vinifera*, powders of *Emblica officinalis*; and oils of *Triticum sativum*.

Composition

Each Oxiwell capsule contains

Extracts

Amra (*Mangifera indica*) ---------- 84 mg

Ashvagandha (*Withania somnifera*) ---------- 78 mg

Yashtimadhu (*Glycyrrhiza glabra*) ---------- 39 mg

Draksha (*Vitis vinifera*) ---------- 27 mg

Powders

Amalaki (*Emblica officinalis*) ---------- 138 mg

Oil

Godhuma (*Triticum sativum*) ---------- 5.5 mg

METHODS

The antioxidant activity of Polyherbal Formulation (PHF), Oxiwell were proved by different *In-vitro* methods which are given as follows -

A. DPPH free radical scavenging activity.

B. Nitric oxide radical scavenging (NO) assay.

C. Determination of Total Phenolics.

D. Determination of reducing power.

E. Hydrogen peroxide scavenging activity.

Preparation of test sample solution

The test sample solutions having concentration 100, 300, 500, 750, 1000 µg/ml of the Polyherbal Formulation (PHF), Oxiwell were prepared in dimethyl sulphoxide (DMSO).

DPPH free radical scavenging activity[9]

The synthetic anti-oxidant, such as 2, 2-diphenyl-1-picrylhyrazyl (DPPH), was used as preservatives in foods and food packaging. This anti-oxidant was used to delay the deterioration of food flavours and odours and increase the shelf life of many foods.[9] However, interest is growing internationally for herbal products, such as essential oils, to replace the synthetic anti-oxidants based on their emerging deleterious side effects. The DPPH scavenging activity of the Polyherbal Formulation (PHF), Oxiwell was measured according to the procedure described by Ashvin VD, et al(2007), with some modifications. Radical scavenging activity of Oxiwell against the stable DPPH radical was determined spectrophotometrically. Accurately weighed 4.3 mg of DPPH was dissolved in 3.3 ml of methanol in a test tube. Solution was protected from light by covering with aluminum foil. 150 µl of above solution was taken and diluted up to 3ml with methanol, the absorbance of this

solution was taken immediately at 517 nm on UV spectrophotometer using methanol as blank. This reading was served as control reading.

The antioxidant activities of PHF were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. 20 microliters of various concentrations (100, 300, 500, 750 and 1000µg/ml) of the Oxiwell in dimethyl sulphoxide (DMSO) were put into appropriate tubes and 4 ml of 0.004% methanolic solution of DPPH was added to each tube. After vortexing, the mixtures were incubated for 20 minutes at 37°C., the absorbance of this solution was taken at 517 nm on UV spectrophotometer using methanol as blank. The absorbance was taken in triplicate manner. Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation,[10]

% inhibition= (Absorbance of control-Absorbance of test /Absorbance of control) × 100.

The decrease in the absorbance of the test mixture (due to quenching of the DPPH free radicals) was measured and the percentage inhibition was calculated. The IC 50 (Inhibition Concentration) values were determined as the concentrations of the test mixtures that gave a 50% reduction in the absorbance from a control blank.

B. Nitric oxide radical scavenging (no) assay[11]

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 M) in phosphate–buffered saline (PH 7.4) was mixed with 3ml of various concentrations of polyherbal formulation and incubated at 25^0C for 150 min. The samples from the above were allowed to react with the Greiss reagent. The absorbance of the chromophore which was formed during the diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylenediamine was read at 546nm.[12] The experiments were repeated in triplicates. The percentage scavenging of the nitric oxide radical activity was calculated by the formula which has been given below and the results were computed.

NO Scavenging (%) = [(Absorbance of Control - Absorbance of test Sample) / (Absorbance of Control)] x 100

Determination of Total Phenolics[13]

The total phenol content (TPC) was determined spectrophotometrically using the Folin-Ciocalteu assay.[13] 0.5ml of 1-5 mg/ml of PHF made up with 0.5ml of distilled water was introduced into test tubes followed by 0.5 ml Folin-Ciocalteu's reagent and gently mixed. The solution was then kept at dark for 5 min and then 1 ml sodium carbonate (7.5% w/v) was added. The tubes were covered with parafilm and kept again in the dark for 1 h. Absorption at 765 nm was measured with a spectrophotometer UV-vis (Jasco V-630) and compared to a gallic acid calibration curve. Each assay was carried out in triplicate. The concentration of total phenolics is expressed as milligram of gallic acid /g of mixture**.**

Determination of reducing power[14]

Assay of Reducing Power was carried out by potassium ferricyanide method. 2.5 ml of various concentrations of polyherbal formulation were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [K3Fe (CN6)] (10g/L). The mixture was then incubated at 50°C for 20 minutes. To this mixture 2.5 ml of trichloroacetic acid (100g/L) was added, which was then centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 ml of the supernatant solution was collected and mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/L) and absorbance was measured at 700 nm. Ascorbic acid was used as standard and phosphate buffer as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

% Reductive ability = Absorbance of Control- Absorbance of Sample/ Absorbance of control x 100

Hydrogen peroxide scavenging activity[14]

Hydrogen peroxide 2mm/L solution prepared with standard (PO4 buffer pH- 7.4). Different concentrations of PHF (100, 300, 500, 750, 1000 µg/ml) prepared in distilled water.1ml of solution of different concentrations of extract (100, 300, 500, 750, 1000 µg/ml) mixed with 0.6 ml hydrogen peroxide solution. After 10 min Absorbance was measured at 230 nm against blank solution containing PO4 Buffer without hydrogen peroxide.

STATISTICAL ANALYSIS

The results were expressed as % Inhibition as Mean ±SD of 3 determinations, on applying test for significance $p<0.05$ was considered as statistically significant. IC 50 values were also calculated for each assay.

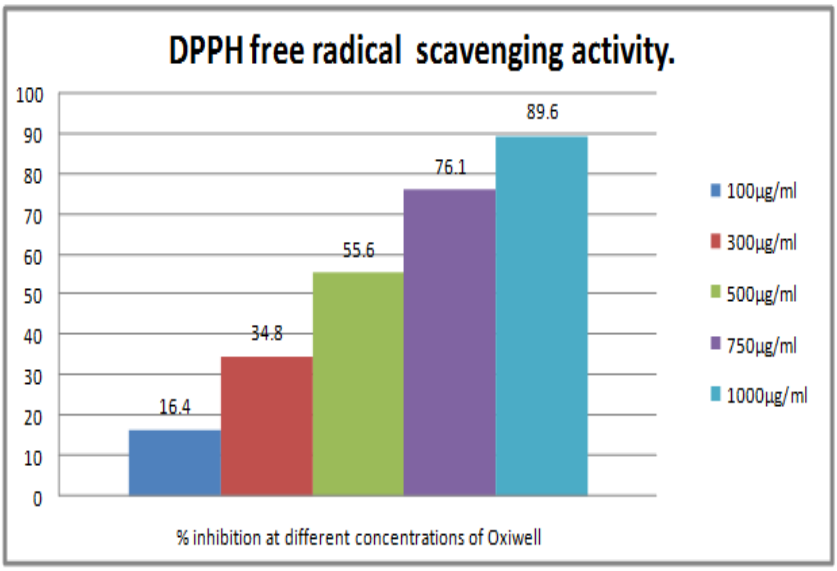
RESULTS AND DISCUSSION

DPPH free radical scavenging activity

The potential to scavenge the DPPH free radical of the PHF, Oxiwell was not significant with the various assayed concentrations of 100-1000 µg/ml. The inhibitory concentration (IC50) of the PHF is found to be 415.92 µg/ml, which was not significant as compared to that of the positive control, which was 84.4 µg/ml.

Table 1: DPPH free radical scavenging activity.

Method	Percentage inhibition at different concentrations				
	100µg/ml	300µg/ml	500µg/ml	750µg/ml	1000µg/ml
DPPH free radical scavenging activity.	16.40±1.40	34.80±1.20	55.60±1.20	76.10±1.30	89.60±1.20



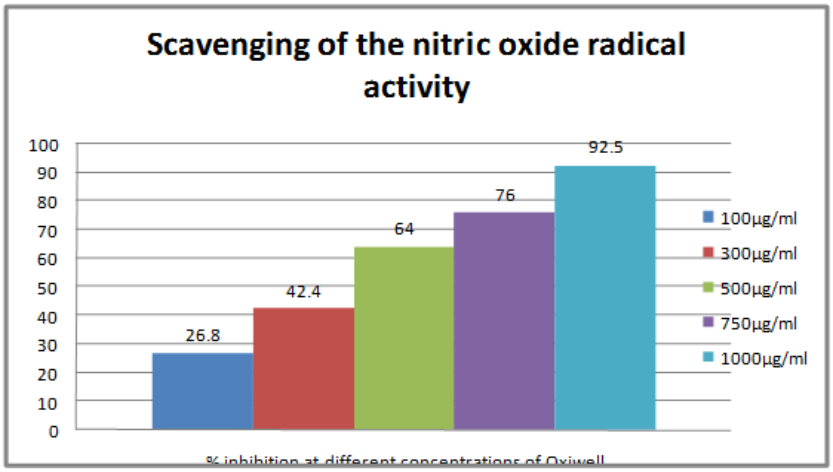
Graph-1: DPPH free radical scavenging activity.

Scavenging of the nitric oxide radical activity

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc and is involved in the regulation of various physiological processes. Excess concentration of nitric oxide is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals. The PHF, Oxiwell revealed that a potential significant inhibition of the nitric oxide free radical scavenging activity, which was mainly concentration dependent with the inhibitory concentration (IC50), at which there was a 50% free radical inhibition, was found to be 400 µg/ml.

Table 2: Scavenging of the nitric oxide radical activity.

Method	Percentage inhibition at different concentrations				
	100µg/ml	300µg/ml	500µg/ml	750µg/ml	1000µg/ml
Scavenging of the nitric oxide radical activity	26.8± 0.10	42.4 ±0.08	64.0± 0.30	76.0 ±0.60	92.5± 1.02



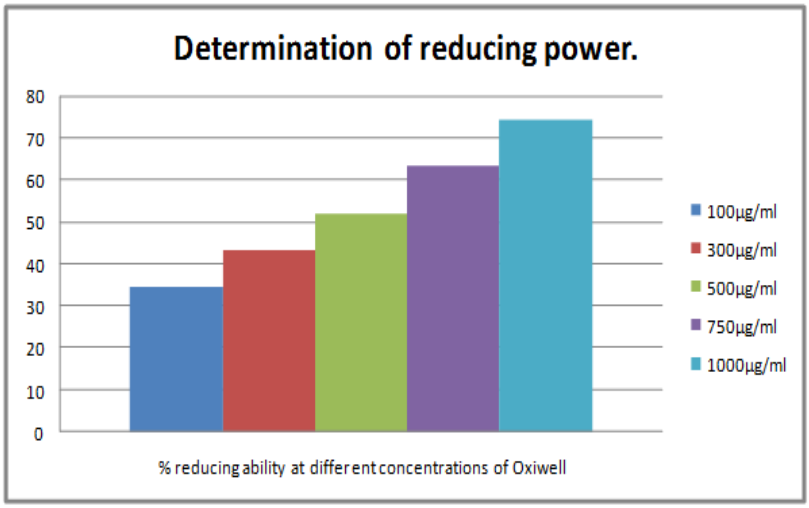
Graph 2: Scavenging of the nitric oxide radical activity.

Determination of reducing power

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The reducing power increased with increasing amount of the extract of polyherbal formulation. The extract of the polyherbal formulation showed the highest reducing ability of 74.41±1.67% at 1000 µg/ml.

Table 3: Determination of reducing power.

Method	Percentage reducing ability at different concentrations				
	100µg/ml	300µg/ml	500µg/ml	750µg/ml	1000µg/ml
Determination of reducing power.	34.56±0.96	43.12±1.81	52.13±1.20	63.40±1.20	74.41±1.67



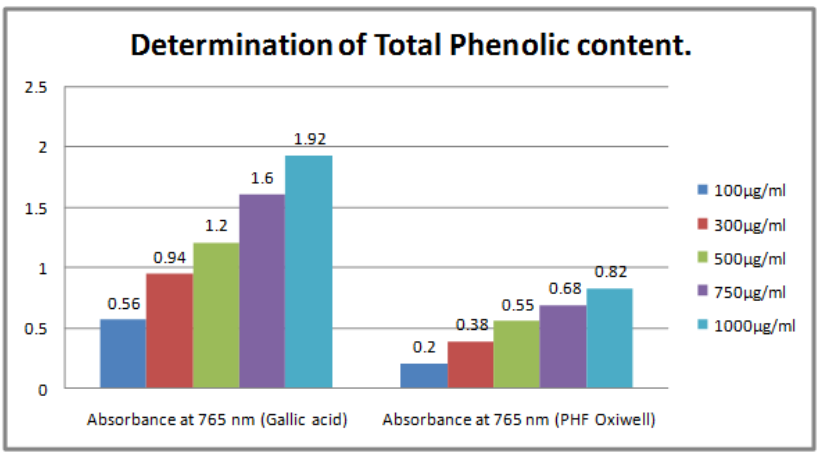
Graph 3: Determination of reducing power.

Determination of Total Phenolic content

In estimation of total phenolic content, when the concentration was increased, absorbance increased in both gallic acid and Polyherbal formulation.

Table 4: Determination of Total Phenolic content.

Methods	Absorbance at 765 nm				
	100µg/ml	300µg/ml	500µg/ml	750µg/ml	1000µg/ml
Gallic acid	00.56	00.94	01.20	01.60	01.92
PHF Oxiwell	00.20	00.38	00.55	00.68	00.82



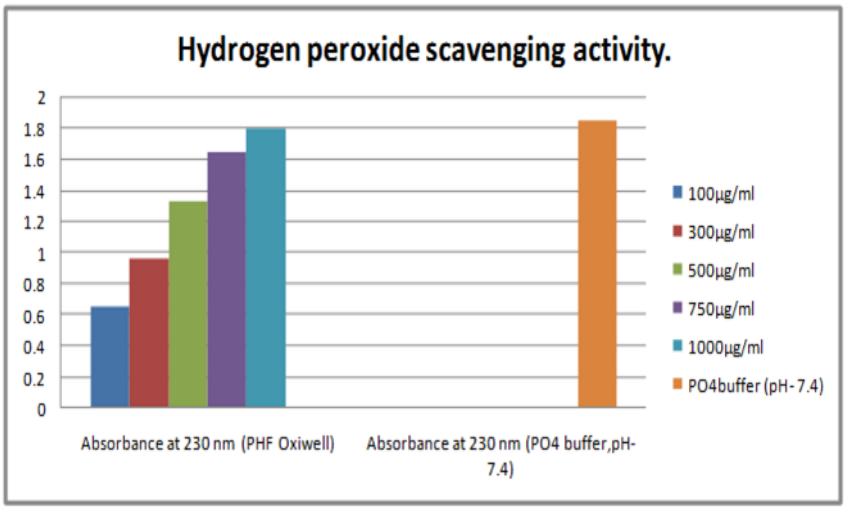
Graph-4: Determination of Total Phenolic content.

Hydrogen peroxide scavenging activity

In determination of Hydrogen peroxide scavenging activity, when the concentration of PHF was increased, absorbance increased.

Table-5. Hydrogen peroxide scavenging activity.

Method	Standard	Polyherbal formulation(Oxiwell)				
	PO4buffer (pH- 7.4)	100µg/ml	300µg/ml	500µg/ml	750µg/ml	1000µg/ml
Absorbance at 230 nm	01.85	00.65	00.96	01.33	01.64	01.80



Graph 5: Hydrogen peroxide scavenging activity.

DISCUSSION

Oxiwell is recommended in the management of oxidative stress associated with Coronary artery disease, Diabetes mellitus, Oral submucous fibrosis and other conditions by inhibiting oxidation reactions, thereby preventing the damage caused by free radicals.

The present investigation was performed to test the hypothesis "Does the PHF, Oxiwell have any antioxidant activity by which it controls oxidative stress associated with Diabetes mellitus, Oral submucous fibrosis and other pathological conditions. The alterations in the oxidant and the antioxidant profile are known to be involved in the pathophysiology, thus affecting the cell and its components, causing damage to them and releasing their products as markers.[16] Many earlier studies have shown the influence of lipid peroxidation, oxidative stress and the antioxidant status on human body.[17-22]

The above data has shown that this PHF, Oxiwell have the antioxidant potential by which it may controls oxidative stress associated with Diabetes mellitus, Oral submucous fibrosis and other pathological conditions.

CONCLUSION

The results of the current study suggests that the PHF, Oxiwell which contains medicinal herbs such as the extracts of *Mangifera indica*, *Withania somnifera*, *Daucus carota*, *Glycyrrhiza glabra*, *Vitis vinifera*, powders of *Emblica officinalis* and *Yashada bhasma*; and oils of *Triticum sativum*, have good antioxidant potential. Thus, it may be concluded on the basis of the current study that the medicinal herbs such as *Mangifera indica*, *Withania somnifera*, *Daucus carota*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Emblica officinalis*, *Yashada bhasma*; and *Triticum sativum* may play an important role in the management of oxidative stress associated with Coronary artery disease, Diabetes mellitus, Oral submucous fibrosis and other conditions by inhibiting oxidation reactions. The current study appears to be the first to investigate antioxidant potential of the poly herbal formulation Oxiwell from The Himalaya Drug Company with respect to its total phenolic content, DPPH free radical scavenging activity, Nitric oxide radical scavenging activity, Hydrogen peroxide scavenging activity, reducing power, and oxidative stability. Thus, further studies are needed to confirm the result of the current study and to evaluate the effects of *Mangifera indica*, *Withania somnifera*, *Daucus carota*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Emblica officinalis*, *Yashada bhasma*; and *Triticum sativum* extracts in oxidative stress.

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