Research Article

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ANTIOXIDANT ACTIVITY OF VARIOUS BIOACTIVE POLYPHENOLIC FRACTIONS FROM INDIGENOUS MEDICINAL PLANTS

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ABSTRACT

Oxidative properties of oxygen play a vital role in diverse biological phenomena. Free radicals are well documented for playing a dual role in our body as both deleterious and beneficial species. Many diseases associated with oxidative stress are now linked to free radicals. Polyphenols and flavonoids comprise the most ubiquitous class of "alternative" antioxidants and protect against free radicals in a number of ways. The aim of the present research study was to evaluate the *in vitro* antioxidant activity of plant extracts rich in polyphenols. The methanolic extract of *Vitis vinifera, Oroxylum indicum and* Policosanol isolated from *Saccharum officinarium* were prepared and evaluated for their primary phytochemical analysis for total phenolic content and *in vitro* antioxidant activity study by DPPH free radical scavenging activity, hydroxyl radical scavenging activity, super oxide radical activity and nitric oxide scavenging activity. Results indicate that all extracts have marked amount of total phenols which could be responsible for the antioxidant activity.

Key words: Reactive oxygen species (ROS), Reactive nitrogen species (RNS), Antioxidants

I. INTRODUCTION:

Oxygen is an element obligatory for life. Living systems have evolved to survive in the presence of molecular oxygen for most biological functions. Oxidative properties of oxygen play a vital role in diverse biological phenomena [1, 2]. Oxygen has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are described free radicals and other non-radical reactive derivatives [3, 4]. ROS and RNS includes radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide

(NO2 •) and lipid peroxyl (LOO•); and non radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) that are produced by aerobic organisms and can easily react with biological molecules including proteins, lipids, DNA thereby causing pathological disorders in the body [5, 6].

Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases including hyperlipidemia and other cardiovascular complications [7].

It is widely accepted that lipid peroxidation plays a central role in the development of cardiovascular diseases, and that low density lipoprotein oxidation is considered to be the hallmark of early atherosclerosis [8, 9]. In our earlier studies the extracts of *Vitis vinifera*, *Oroxylum indicum* and isolated policosanol from *Sacchrum offiinarium* have demonstrated significant antihyperlipidemic activity. Hence the objective of the present research study was to evaluate the antioxidant and free radical scavenging activity of these plant extracts against different radicals and understand their role in prevention of atherosclerosis and other congestive heart diseases.

II.METHODS/ DESIGN:

Collection of plant material and preparation of extracts:

The medicinal plants selected for the study included *Vitis vinifera*, *Oroxylum indicum* and Policosanol isolated from sugarcane waste. The bark of *Oroxylum indicum was* procured from local market at Paydhuni, Mumbai and was authenticated at Nicholas Piramal India Ltd, Goregaon, Mumbai, while the other two plants *Vitis vinifera* and *Saccharum officinarum* which are commonly available were authenticated at the laboratory of Pharmacognosy & Phytochemistry, Prin. K. M Kundnani College of Pharmacy, Mumbai and used for the study.

The voucher specimens have been maintained at the department. The dried materials of the bark of *Oroxylum indicum,* fruits of *Vitis vinifera,* were powdered to facilitate proper extraction and then subjected to soxhlet extraction using methanol as a solvent. The methanolic extracts were concentrated under vacuum and used for the final study [10, 11].

Isolation of policosanol from sugar cane waste:

Calcium hydroxide dissolved in 7 L of water was added to sugar cane wax--previously

melted at 100.degree.-120.degree. C. The saponification process was continued for 8 to 10 hours with stirring. The compound isolated was further extracted using methanol as solvent for 12 hours by soxhlet extraction. The obtained extract is left to cool at room temperature, and recrystallized in dichloromethane to obtain a purity of 93.77%. The melting point of the mixture ranges from 80.0.degree.-82.0.degree. C [12].

EXPERIMENTAL:

1.0.1 Materials and Methods:

1, 1- Diphenyl 2- picryl hydrazyl, Greiss reagent, Deoxyribose, Quercetin, Resveratrol, Nitro blue tetrazolium, were obtained from Sigma Aldrich, USA Titanium chloride, dimethyl sulphoxide (DMSO), ferric sulphate, ascorbic acid, EDTA, trichloroacetic acid, histidine, sodium hypochiorite, N. N dirnethyl paranitrosoaniline were Analar grade obtained from S.D. Fine Chemicals Mumbai. All other chemicals were of laboratory grade. Oxyresveratrol was obtained as a gift sample from Aasha Biochem, Bangalore, Karnataka.

1.0.2 Instruments:

Spectrophotometric measurements were performed with UV Visible spectrophotometer (Jasco UV 550)

1.0.3 Preparation of test samples:

The various plant extracts obtained as described in section II were reconstituted in methanol to obtain a concentration of 1mg/ml. A spray died powder of *Vitis vinifera* was also used for the study quantified in terms of its gallic acid and catechin content.

1. TOTAL PHENOLIC CONTENT:

The total polyphenolic content of the various extracts was determined using *Folin cio-calteau* method. To 1ml of extract, 1 ml *Folin cio-calteau* reagent was added. To this solution 1 ml of sodium carbonate solution was added after 2 minutes. The solution turns to blue color forming a complex. The test tubes were centrifuged at 2000 rpm. The supernatant was collected and the absorbance readings were taken at 765 nm. Calibration curve of gallic acid was prepared using different concentrations of gallic acid and the same process was followed as discussed above. The total phenolic content was expressed as mg gallic acid equivalent (GAE) / gm on dry weight basis [13].

2. EVALUATION OF RADICAL SCAVENGING ACTIVITY:

A. Hydroxyl Radical Scavenging activity:

The reaction mixture consisted of 3mM Deoxyribose, 0.1mM ferric chloride, 0.1mM EDTA, 0.1mM Ascorbic acid, 3mM Hydrogen peroxides in Phosphate Buffer (pH – 7.4). To this reaction mixtures various concentration of test extracts were added. After incubation for 30 minutes at 37^0 C, 5% TCA and 1% TBA were added. The Reaction mixture was kept in boiling water bath for 30 minutes. The test tubes were allowed to cool and absorbance readings were measured at 532 nm [14].

B. Singlet Oxygen radical Scavenging activity:

The assay mixture contained 45mM sodium phosphate buffer (pH7. 1). 10 mM histidine, 10mM sodium hypochlorite, 10mM hydrogen peroxide, 50mM N, N dimethyl paranitrosoaniline and different concentrations of the extracts. The total reaction volume was made to 2 ml and incubated at 30° C for 40 minutes.

The extent of singlet oxygen production was determined by measuring the decrease in absorbance of N, N dimethyl paranitrosoaniline at 440 nm. Relative scavenging activity (% inhibition production of singlet oxygen) of the extracts was estimated from the difference in absorbance of N, N dimethyl para nitrosoaniline with and without addition of the extracts [15].

C.DPPH Free radical Scavenging activity:

The free radical scavenging potential of the test extracts was assessed by adding 50ul of methanolic solution of DPPH (2mg/ml) to the test extracts dissolved in methanol at various concentrations. The decrease in absorbance was read at 516nm at room temperature. Percentage reduction in absorbance was calculated from initial and final absorbance of each level [16].

D. Superoxide radical Scavenging activity:

The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, NBT 0.1 mg/3 mL, added in that sequence. The reaction was started by illuminating the reaction mixture with different concentrations of sample extract and the production of blue farmazone, the absorbance was measured at 590 nm and IC_{50} was calculated. Methanol was used for blank reading [17].

E. Nitric oxide radical Scavenging activity:

Nitric oxide scavenging activity was measured by the spectrophotometer method. Sodium nitroprusside (5 mmol) in phosphate-buffered saline was mixed with a control without the test compound, but with an equivalent amount of methanol. Test solutions were added at different concentrations in methanol and incubated at 25°C for 30 minutes. After incubation, 1.5 ml of incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm [18].

F. Assesment of Pyrogallol Red Bleaching by Peroxy nitrite:

Pyrogallol (PR 100 µmol/L final concentration) was dissolved in K_2HPO4-KH_2PO4 buffer. Test samples were added into the PR solution and incubated at room temperature for 10 min; addition of ONOO- (200 µmol/L) or HOCl (125 µmol/L) followed and the mixture was vortexed immediately. The decrease in absorbance at 542 nm was determined [19]

2. Evaluation of Antioxidant Activity by Lipid Peroxidation Method:

The antioxidant activity of plant extracts was evaluated by calculating inhibition of lipid peroxidation .which was performed using liver homogenate of mice. Mice liver homogenate (10%) was prepared by homogenizing the fresh liver in 0.15 M KCl solution. This fresh liver homogenate was mixed with 0.15 M KCL and tris hydrochloride buffer. The various extracts of *Vitis vinifera, Oroxylum indicum* and Policosanol at different concentrations were then added. Quercetin at various concentrations was used as standard. *In vitro* lipid peroxidation was initiated by addition of 100 µM ferrous sulphate and 100 µM Ascorbic acid. After incubation for 1 hour at 37 degrees, the reaction was terminated by addition of thiobarbituric acid and then boiled at 95 degrees for 15 minutes for development of colored complex. On cooling test tubes were centrifuged at 4000 rpm for 10 minutes. Absorbance of supernatant was determined colorimetrically at 532 nm and percent inhibition of TBARs formation was calculated with respect to control in which no test sample was added. The IC_{50} values were calculated for all test

material by subjecting the results to linear regression [20, 21].

RESULTS AND DISCUSSION:

In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicine. Recently, many natural antioxidants have been isolated from different plant materials [22]

The present study corroborates and extends previous findings indicating some extracts rich in polyphenols and their isolates as promising antioxidant pharmacophores. The scavenging activities for ROS/RNS shows promising results, considering the micromolar range of the IC_{50} values found. The antioxidant activity has been studied by using gallic acid as the standard for phenolic content measurement, and shows promising results. Therefore, it is clear that all plant extracts have good antioxidant activities as well as high polyphenolic contents.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH both transfer electron or hydrogen atom to DPPH thus neutralizing its free radical character and convert it to 1 – 1 diphenyl – 2 – picryl hydrazine and the degree of discoloration indicates the scavenging activity of the test samples. Hence DPPH is usually used as a substance to evaluate the antioxidant activity. The results show that the extract of *Vitis vinifera* has antiradical activity by inhibiting DPPH at IC_{50} value of 48.25 µg/ml, followed by the extract of *Oroxylum indicum* IC_{50} value of 57.60 µg/ml, followed by policosanol IC_{50} value of 64.40 µg/ml as compared to standard hydroxyl radical scavenger quercetin IC_{50} value of 12.94 µg/ml. It establishes the fact that the extracts posses hydrogen donating abilities to act as antioxidants.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and are responsible for cellular damage. They were produced in this study by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH. The extracts showed strong scavenging activity of hydroxyl radicals which could inhibit lipid damage. The IC_{50} value indicates that the

extract of *Vitis vinifera* showed maximum activity followed by the extract of *Oroxylum indicum* followed by policosanol as compared to standard hydroxyl radical scavenger quercetin which indicates an IC_{50} value of 22.08 µg/ml.

One of the main sources of superoxide radical ($^{-}O_2$) is the enzyme xanthine oxidase (XO). Superoxide anion is also very harmful to cellular components. It has already been establish reports that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. Superoxide free radical was formed by alkaline DMSO which reacts with nitro blue tetrazolium (NBT) to produce colored diformazan. The results indicate that all extracts scavenge superoxide radical thereby inhibiting formazan formation. Addition of extract of *Vitis vinifera* showed maximum activity IC_{50} value 41.86 µg/ml followed by the extract of *Oroxylum indicum* IC_{50} value 53.67 µg/ml followed by policosanol IC_{50} value 71.82 µg/ml as compared to standard quercetin IC_{50} value of 16.39 µg/ml.

It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. Nitric oxide is the principal radical responsible for atherosclerosis and congestive heart diseases [23, 24, 25]. The present study proved that the extract of *Vitis vinifera* showed best activity more potent nitric oxide scavenging activity than the standard quercetin followed by the extract of *Oroxylum indicum* followed by policosanol. This could be one possible reason to explain the prophylactic benefit of *Vitis vinifera* extract in prevention of atherosclerosis.

Peroxynitrite ($ONOO^-$) is relatively stable compared to other free radicals but once protonated it forms the highly reactive peroxynitrous acid (ONOOH) Generation of excess $ONOO^-$ leads to oxidative damage and tissue injury. According to the present results, all the plant extract inhibits Evans Blue bleaching by scavenging peroxynitrite and its activity is greater than that of the reference quercetin. Singlet oxygen is

generated in the body by ultraviolet radiation. Singlet oxygen induces hyperoxidation and oxygen cytotxicity and decreases antioxidative activity. The present study of *Vitis vinifera* showed maximum activity followed by the extract of *Oroxylum indicum* followed by policosanol.

It is widely accepted that lipid peroxidation plays a central role in the development of cardiovascular diseases, and that low density lipoprotein oxidation is considered to be the hallmark of early atherosclerosis [26]. Oxidized LDL (Ox- LDL) is atherogenic as it causes arterial cell death, accumulation of inflammatory cells in the arterial wall, and stimulation of growth factors and cytokine release. In addition, Ox-LDL contributes to platelet aggregation, smooth muscle cell proliferation and thrombotic and inflammatory processes. Free-radical mediated lipid peroxidation proceeds by a chain mechanism, where the lipid peroxyl radicals act as chain-carrying species. The mechanism and products of free-radical-mediated lipid peroxidation are independent of the initiating species, since the chain propagation is mediated by lipid peroxyl radicals, regardless of whether the oxidation is initiated by attack of hydroxyl or superoxide radical, peroxynitrite, tyrosyl radical or other radical species [27, 28]. The present study reveals that extracts of *Vitis vinifera* and *Oroxylum indicum* shows inhibition of thiobarbituric acid reacting substances (TBARs) followed by policoosanol.

The results indicate that *Vitis vinifera plant* extract contains significant amounts of flavonoids and phenolic compounds followed by *Oroxylum indicum* and policosanol which could be one of the reasons contributing higher antioxidant activity. The mechanism by which phenolic compounds are able to scavenge free radicals is yet to be exactly established. In any case it seems to be clear that the basic structure of compounds and other structural factors are very important in the scavenging mechanism. Literature cites that the aromatic OH groups are the reactive centers, primarily dihydroxy catechol group, and their activity can be enhanced by electron donating effects of other substituent. For flavonoid compounds, O-dihydroxy groups in the B-ring, the presence of a C-2, 3 double bond in conjunction with 4-oxo in the C-ring. As regards to the antioxidant activity of trans-resveratrol, a stilbene compound, the presence of the catechol structure and the presence of the meta-hydroxy structure in ring A seem to be essential criteria. Therefore, quercetin, and

resveratrol were the most active peroxynitrite, superoxide and hydroxyl radical inhibitors as compared to 5, 6, 7 trihydroxy flavone. The above results indicate the maximum radical scavenging activity is shown by resveratrol a stilbene isolated from the extract of *Vitis vinifera* followed by *Vitis vinifera* spray dried powder that is known to contain maximum amount of polyphenols and flavonoids perhaps due to the presence of catechin and epigallocatechins and other proanthocyanidins.

This confirms the previously referred importance of the hydroxyl group at C-3, which distinguishes quercetin (a flavonol) from 5, 6, 7 trihydroxy flavone (a flavone), in their radical scavenging activity potencies. This structural feature provides the conjugation between B-ring and C-ring, and contributes to the stabilization of the phenoxy radical. This fact points to a likely contribution of the styryl moiety to the molecular stabilization, increasing the compound's antiradical activity.

The singlet oxygen scavenging activity of flavonoids seems to depend particularly on the OH-substitution pattern in B-ring. Compounds with a 3',4'- dihydroxyl pattern presented a considerably higher effect than those lacking this feature. Nevertheless, the number and position of the OH substituent's in the A-ring also contributes to the singlet oxygen scavenging activity. An OH group in C5 seems to positively affect the scavenging behavior of the antioxidant molecule, while this substitution in C7 appears to bring no advantage [29].

The high peroxyl (ROO^{-}) scavenging activity shown by some of the studied compounds may be of extreme value, since during its sustained overproduction, the walls of affected tissue cells involved become amenable to lipid peroxidation, explaining the increase of lipid peroxidation marker compounds in oxidative stress related diseases. The results obtained in that same study showed that the B-ring catecholic derivatives were more effective.

Thus, the 4-hydroxyl group in B-ring also seems to be of extreme importance for $ONOO^{-}$ and the absence of an OH substituent in the B-ring drastically decreases the scavenging effect. A possible explanation for this fact might be on the contribution of the styryl moiety to the stabilization of the phenoxy radical that is formed during the scavenging reaction. NO^{-} scavenging activity depends mostly on the hydroxylation pattern of the B-ring. The compounds with a 3', 4'catecholic group were shown to be very potent protectors of

the NO^-dependent oxidation [30]. The presence of antioxidant activity is not the only contributing factor in atherosclerosis. Further studies pertaining to their *in-vivo* activity needs to be carried to determine the exact mechanisms as to how they could act in the prevention and etiogenesis of diseases. Some of the studied plant extracts and their isolates were shown to be remarkable scavengers of those reactive species. Clearly, the therapeutic use of the tested extracts and their isolates still requires a battery of *in vitro* and *in vivo* toxicological assays, to confirm their efficacy and assure their safety.

RESULTS OF SCAVENGING ACTIVITIES OF PLANT EXTRACTS ON VARIOUS RADICALS IC_{50} (µg/ml)

IC 50 VALUES µg/ml						
Extracts	**Hydroxyl**	**Superoxide**	**Singlet oxygen**	**DPPH**	**Nitric oxide**	**Peroxy nitrite**
Vitis vinifera	34.41	41.86	57.69	48.25	23.37	28.76
Oroxylum indicum	40.28	53.67	62.03	57.60	38.78	45.37
Policosanol	62.18	71.82	78.24	64.40	64.39	79.64
Vitis vinifera Spray dried	24.19	32.37	43.57	40.05	15.07	20.78
Quercetin	22.08	16.39	36.29	12.94	26.12	11.43
Resveratrol	16.53	14.38	23.82	4.16	20.15	10.75
5, 6, 7 Trihydroxy flavone	38.97	56.93	46.37	23.62	38.74	59.36
Oxyresveratrol	17.25	12.31	28.73	3.07	12.48	6.47

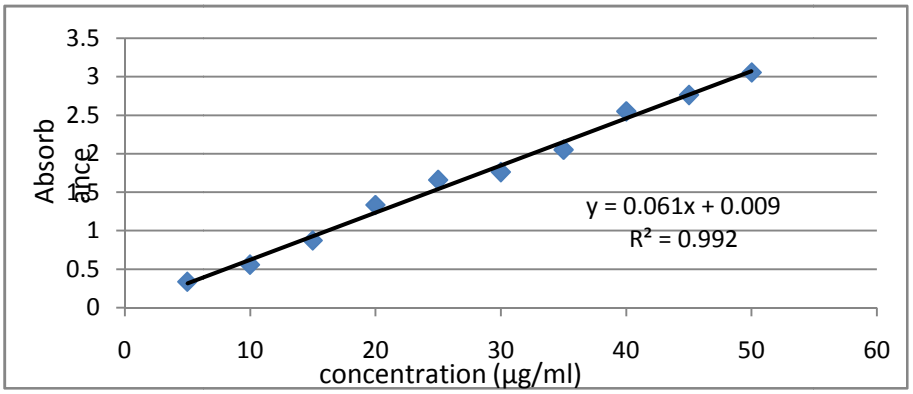
TABLE-2: IC50 VALUES OF TOTAL EXTRACTS AND THEIR ISOLATES FOR LIPID PEROXIDATION INHIBITORY ACTIVITY.

Extracts µg/ml	**IC_{50} µg/ml**
Vitis vinifera	50.94
Policosanol	74.85
Oroxylum indicum	61.27
Quercetin (STANDARD)	38.13
Vitis vinifera Powder (SD)	45.84

TABLE-3: RESULTS OF TOTAL PHENOLIC CONTENT OF VARIOUS EXTRACTS

Sr. No	Extract	Total Phenolic content (mg GAE/gm)
1.	*Vitis vinifera*	43.58
2.	*Oroxylum indicum*	30.57
3.	*Vitis vinifera* spray dried powder	79.85

STANDARD CURVE OF GALLIC ACID



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