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Research Article

SYNERGISTIC ACTION OF PHYTOCHEMICALS AUGMENTS THEIR ANTIOXIDATIVE EFFICACY: AN IN VITRO COMPARATIVE STUDY

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ABSTRACT

Objective: The present study was aimed to find out the comparative *in vitro* antioxidative activity of three commercially available polyherbal formulations (PHFs) and *Gymnema sylvestre,* the major constituent of all the test formulations.
Methods: Different *in-vitro* free radical scavenging assays such as 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Nitric oxide (NO), reducing power assay and ferrous sulphate ($FeSO_4$) induced lipid peroxidation (LPO) in liver and kidney homogenates were performed in four parallel sets for each drug. Total polyphenols and flavonoid contents were also estimated.
Results: Although all the test drugs exhibited significant free radical scavenging activity, Madhuhari was found to be the most effective one against DPPH, NO and $FeSO_4$ induced free radical systems. However, their higher concentrations were peroxidative in nature.
Conclusion: Poly herbal formulations were more antiperoxidative as compared to mono herb preparation. As all the test drugs showed decreased efficacy and/or peroxidative effects at their higher concentrations, their higher dose should be avoided. Significant amount of polyphenols and flavonoids present in the test drugs supported their antioxidative property. This study appears to be the first one to demonstrate a comparative protective as well as harmful effect(s) at higher concentration of three herbal formulations.

Keywords: Antioxidants, DPPH, harmful effects, herbal drugs, lipid peroxidation

INTRODUCTION

Increased oxidative stress is believed to generate free radicals which in turn contribute in the development of different diseases like atherosclerosis, arthritis, ischemia, cancer and diabetes mellitus [1,2]. In fact, oxidative stress can produce major derangements of cell metabolism such as DNA- strand break, damage to membrane ion transporters and other specific proteins and lipid peroxidation (LPO) [3]. LPO usually refers to the oxidative degradation of lipids, especially polyunsaturated fatty acids (PUFAs) that are more susceptible for free radical attack. Cells are rich source of PUFAs, hence are readily oxidized. Moreover, self propagating nature of LPO makes it more deadly and highly damaging [4].

Herbal extracts, which are known as rich source of antioxidant, the phytochemicals can break chain propagation process of oxidative damage by providing proton or an electron to free radical [5]. In fact, plant derived secondary metabolites such as polyphenols, flavonoids, alkaloids and saponins show various biological and pharmacological antioxidative properties and promote health benefits [6]. Because of the known antioxidative potential of many plants [7], more attention is being given on the investigations in herbal medicines. Herbs like *Momordica charantia, Eugenia jambolana*, *Tinospora cardifolia Allium sativum*, *Ocimum sanctum* [8,9], *Phyllanthus amarus, Withania somnifera* and *Gymnema sylvestre* [10] with significant antioxidative properties are well known antidiabetic herbs. It is known that antioxidants which inhibit LPO *in vitro* may exert similar affects in *in vivo* [3]. Very often it is also believed that these natural supplements do not exhibit side effects. However, recently new interest has also been generated on the side effects of herbal preparations [11] and some reports indicated adverse health effects of individual herbs [12,13]. Even pro-oxidative/harmful effects of pharmacological combinations which may appear due to herb-herb interactions are known in both *in vitro* and *in vivo* systems [14-16]. Numerous case series have also been published on adverse events of different herbal formulations which are sold as over-the-counter drugs [17, 18]. Along with these issues, safety of herbal drugs has become a serious question, as a number of adverse effects have been reported including hepato-toxicity, renal failure, cardiovascular dysfunction and allergic reactions [8,11,14,19,20]. Therefore, the main aim of our study was to evaluate the relative antioxidant activities of marketed polyherbal formulations (PHFs) and to compare the effects with that of *Gymnema sylvestre*, which is a major constituent of all tested formulations, Diabecon, Madhuhari and Madhumehari that are commonly used as antidiabetic PHFs. Number of reports showed antihyperglycemic property of Diabecon as single drug or in combination with glibenclamide in animals and human beings [21]. However, none of these reports explained the possible involvement of antioxidative actions of the PHFs. Therefore, it was imperative to reveal concentration dependent anti-lipid peroxidative and free radical scavenging activities of some antidiabetic PHFs, if any.

Since *in vitro* study permits an enormous level of simplification of system under study [22] and similar results can be predicted for *in vivo* study, *in vitro* testing was done to evaluate the hitherto unknown comparative efficacy of three different antidiabetic poly herbs on their antiperoxidative property.

MATERIALS AND METHODS

Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA ;while Greiss reagent, sodium nitroprusside, phosphoric acid and thio-barbituric acid (TBA) were supplied by Hi Media Laboratories Ltd., Mumbai, India. Malondialdehyde (MDA), sodium dodecyl sulphate (SDS), acetic acid, and all other reagents were purchased from E-Merck (India) Ltd., Mumbai, India. Polyherbal drugs, Diabecon (Himalaya Pvt. Lmt., Bangalore-560058, India. Batch No.37200283B), Madhuhari (Shivayu ayurveda, Nagpur- 441107, India. Batch No. MHPA / 1114) and Madhumehari (Baidhyanath Co., Nagpur- 441107, India. Batch No. 110060) and *G. sylvestre* extract were purchased from authorized medical store of local market, Indore and were used as 0.5% carboxy methyl cellulose (CMC) suspension, 70% ethanolic extract (for first two) and aqueous extract for last two. Details of the herbal composition of the three polyherbal formulations are mentioned in table 1.

Animal ethics

Standard ethical guidelines of the committee for the purpose of control and supervision on experiments in animals, ministry of environment and forest, Govt. of India, were followed. The approval of departmental ethical committee for handling and maintenance for experimental animals was also obtained before starting the experiments.

Preparation of tissue homogenate

Two overnight fasted healthy Wistar rats were sacrificed after anaesthetizing with mild chloroform. Liver and kidney were excised immediately and washed with chilled phosphate buffer saline (PBS-0.1 M, pH 7.4). These organs were further chopped and homogenized in PBS to obtain 10% w/v homogenate and used within one hour for further experimentation.

Table1: Composition (different herbal contents) of three test drugs

S.N.	Plant's name	Diab-con	Madh-uhari	Madhum-ehari
1	*Gymnema sylvestre*	30	90	40
2	*Momordica charantia*	20	40	40
3	*Eμgenia jambolana*	20	40	-
4	*Tinospora cardifolia*	10	40	40
5	*Asphaltum punjabinum*	30	10	40
6	*Curcuma longa*	10	-	20
7	*Acacia arabica*	-	40	-
8	*Curcuma amada*	-	40	-
9	*Aegle marmelos*	-	40	-
10	*Azadirachta indica*	-	20	8
11	*Picrorrhiza kurroa*	-	20	20
12	*Trigonella foenum gracecum*	-	20	20
13	*Sygium cumini*	-	-	40
14	*Acacia catachu*	-	-	20
15	*Emblica officinalis*	-	-	40
16	*Pterocarpus marsupium*	20	-	20
17	*Ficus bengalensis*	-	-	20
18	*Abhrak bhasma*	10	-	8
19	*Swarna makshik bhasma*	-	-	8
20	*Cinnamomum tamala*	-	-	8
21	*Glycyrrhiza glabra*	20	-	-
22	*Casearia esculenta*	20	-	-
23	*Asparagus recemosus*	20	-	-
24	*Boerhaavia diffusa*	20	-	-
25	*Sphaeranthus indicus*	10	-	-
26	*Swartia chirata*	10	-	-
27	*Tribulus terrestris*	10	-	-
28	*Phyllanthus amarus*	10	-	-
29	*Gossipium herbaceum*	10	-	-
30	*Barberis aristata*	5	-	-
31	*Aloe vera*	5	-	-
32	*Piper nigrum*	10	-	-
33	*Ocimum sanctum*	10	-	-
34	*Abutilon indicum*	10	-	-
35	*Rumex maritimus*	5	-	-
36	*Vang bhasma*	5	-	-
37	*Akik pishti*	5	-	-
38	*Shingraf Bhasma*	5	-	-
39	*Yasad bhasma*	5	-	-
40	*Zingiber officinalis*	5	-	-

Quantity of each component is shown in gram / 400 gram dry weight of each drug, as given on the container

Experimental design

Estimation of *in vitro* antioxidant activity

The antioxidative activity of drugs were determined *in vitro* by different free radical scavenging assays in which graded concentrations of test drugs were added with assay mixtures. As no report was available on *in vitro* study of these drugs, we took wide range of concentrations of test drugs in all the assays to find out the maximum inhibitory concentration of each drug. Each experiment was repeated with the most effective concentration of each drug to verify the results.

DPPH assay

Methanolic stock solutions of different concentrations (0.07-1000 µg/ml) of all drugs were prepared. One ml of extract was mixed with 0.5 ml of 0.15 mM DPPH and incubated for 30 min in dark at 20˚C. Control tube was prepared by adding methanol in place of extract. Optical density (OD) was taken at 517 nm. Ascorbic acid was used as standard and percent (%) scavenging activity was determined [23].

NO radical scavenging assay

This assay was performed following the protocol of Wang *et al* [25]. Sodium nitropruside was used as a NO free radical donor *in vitro*. In assay mixture 0.5 ml sodium nitropruside (10 mM in 0.2 M PBS, pH 7.4) was added with 0.5 ml of different concentrations (0.07- 1000 µg/ml) of drugs and incubated for 150 min at 20˚C in dark. Then 1 ml Griess reagent was added to read OD at 542 nm against blank. The NO scavenging activity (%) was measured with respect to control. The nitrite concentration was calculated in relation to the absorbance of a standard solution of sodium nitrite. Results were expressed as percentage nitrite produced with respect to the control, which was devoid of drug.

Reducing power assay

The ferric ion reducing power of the extracts was determined by the method of Lim *et al* [26]. In brief 0.75 ml extract of individual drug (0.3-5000 µg/ml diluted in PBS of 0.2 M, pH 6.6) were mixed with 0.75 ml PBS and 0.75 ml of potassium ferricyanide (1% w/v in DW), followed by incubation at 50˚C for 20 min. The reaction was stopped by adding 0.75 ml 10% TCA and centrifuged at 3000 rpm for 10 min. Then 1.5 ml supernatant was mixed with 1.5 ml DW followed by 1.5 ml of freshly prepared $FeCl_3$ (0.1% w/v) solution. After 10 min, OD was read at 700 nm. Higher OD of reaction mixture indicates greater reducing power. Ascorbic acid was used as standard.

Standardization of concentration of $FeSO_4$ for induction of LPO

LPO was induced following the procedure of Parmar and Kar [8,20] as routinely used in our laboratory. Different concentrations of $FeSO_4$ (1-20 mM) were added to 1 ml (10% w/v) liver tissue homogenate, each in triplicate. In control tubes DW was added in place of $FeSO_4$. The reaction mixture was incubated for 1 hour at 37˚C. MDA formed was estimated by TBARS method. Similar concentrations of $FeSO_4$ were also taken for the study in kidney homogenate [8,20,24].

Estimation of antiperoxidative efficacy of drugs

Considering the most effective $FeSO_4$ concentration (10 mM), antiperoxidative effects of drugs were evaluated following the protocol of Ohkawa et al. [27]. Wide ranges of concentration of drugs were taken in triplicate (1 µg/ml-100 mg/ml as). Tissue homogenate treated with $FeSO_4$ and different concentrations of test drugs were incubated and processed as mentioned earlier [8,20,24]. LPO was measured and expressed in term of MDA formed / h / mg protein. In each experiment four parallel sets of all four drugs were run to evaluate their comparative efficacy.

Estimation of total polyphenols and flavonoids

Total polyphenols of the test extract was measured by the protocol of Leontowicz *et al* using Folin-Ciocalteu [23]. In brief, 0.125 ml of test extract of known concentration (100 mg/ml) was diluted with 0.5 ml of distilled water (DW), followed by the addition of 0.125 ml of Folin-Ciocalteu reagent. The final volume was made up to 3.0 ml with DW and was incubated at room temperature for 90 min. Finally the absorbance was measured against the prepared blank at 765 nm in comparison with standard of known concentrations of gallic acid. The results were expressed in mg gallic acid equivalent / 100 g dry weight of the extract. The coefficient of determination obtained was $r^2 = 0.976$.

Total flavonoids were determined following the method of Leontowicz *et al* [23] with some modification as followed in our laboratory earlier [8,20,24]. In brief 0.25 ml of the extracts in 25, 50 and 100 ppm concentrations were diluted with 1.25 ml DW, followed by the addition of 75 µl of 5% sodium nitrite solution and 150 µl of 10% aluminum chloride ($AlCl_3.6H_2O$). After incubation for 5 min, 0.5 ml of 1M NaOH was added. The final volume was made up to 2.5 ml with DW and OD was taken against the prepared blank at 510 nm in comparison with standards prepared similarly with known concentrations of quercetin. The results were expressed in mg quercetin equivalent/ 100 g dry weight of the extract. The coefficient of determination was, r^2 =0.965. Results are expressed as mg of quercetin equivalents / 100 g dry weight of the extract.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was done considering one–way analysis of variance (ANOVA) followed by unpaired student's t-test using a trial version of prism 6 soft ware for windows (Graph Pad Software, Inc., La Jolla, CA, USA) and *P-value* of 5% and less were considered as significant. EC_{50} values were calculated from the linear regression equation and compared by paired t- test.

RESULTS

Antioxidant activities

All test drugs showed significant antioxidant activity in concentration dependent manner in DPPH, reducing power and NO scavenging assays. However, more than 50% free radical scavenging activities were considered to be effective. Madhuhari was found to have the highest free radical scavenging activity (86%) and lowest EC_{50} values for all above mentioned assays at relatively lower concentration as compared to other three test drugs. (Table 2-4)

Table 2: Scavenging of DPPH free radicals (in %) by different test drugs.

Drugs (µg/ml)	15.25	31.5	62.5	125	250
Diabecon	78.3±0.37^c	82.36±0.95^c	77.33±0.36^a	71.32±0.50	60.23±0.95^c
Madhuhari	67.96±0.59^c	73.30±0.73^b	86.38±0.32^c	85.34±0.23^c	75.60±0.58^c
Madhumehari	40.17±0.56	72.33±0. 87	76.02±0.93	83.12±0.11^c	71.33±0.26^c
G. sylvestre	34.58±0.42	67.24±0.46	71.72±0.56	69.65±0.53	34.52±0.24
Ascorbic acid	87.56±0.05^c	95.55±0.12^c	96.98±0.05^c	96.70±0.55^c	97.53±0.09^c

Data are expressed in % inhibition (mean ±SE of n=3) in comparison to that of the standard, ascorbic acid. ^{a} *P*<0.05; ^{b} *P*<0.01 and ^{c} *P*<0.001 significantly more effective as compared to the respective concentration of *G. sylvestre*

Table3: Scavenging of Nitric oxide (NO) free radicals (in %) by different test drugs

Drugs (mg/ml)	0.62	1.25	2.50	5.0	10
Diabecon	76.44±0.84^a	83.73±0.68^b	89.63±0.39^b	91.26±0.42^b	81.74±0.27^a
Madhuhari	79.03±0.65^a	88.43±0.68^b	93.55±0.57^b	92.16±0.78^b	87.53±0.85^a
Madhumehari	73.37±0.75	78.01±0.78	85.66±0.89	86.08±0.89	84.57±0.79
G. sylvestre	70.23±0.86	75.54±0.73	78.61±0.69	82.58±0.49	77.89±0.67
Ascorbic acid	85.78±0.52^c	86.92±0.94^c	91.74±0.31^c	93.91±0.39^b	93.73±0.39^b

Data are expressed in % of scavenging activity, as compared to that of standard, ascorbic acid (mean ±SE of n=3). Values within a group also differ significantly (*P* ≤ 0.05). ^{a} *P*<0.05; ^{b} *P*<0.01 and ^{c} *P*<0.001 significantly more effective as compared to the respective concentration *of G. sylvestre* .

Table4: Reducing power of different test drugs (in % reduction of OH free radicals)

Drugs (mg/ml)	0.07	0.15	0.3	0.6	1.25
Diabecon	7.30±0.03	10.95±0.27	17.13±0.25^a	26.75±0.66^b	42.24±1.86^b
Madhuhari	34.20±0.26^c	64.31±1.94^c	93.35±1.86^c	100±0.00^c	100±0.00^c
Madhumehari	33.39±0.68	58.30±0.65^c	97.61±0.32^c	100±0.00^c	100±0.00^c
G. sylvestre	5.85±0.08	7.9±0.01	14.71±0.26	33.72±1.55	51.41±2.33
Ascorbic acid	56.57±0.92^c	98.23±0.40^c	100±0.00^c	100±0.00^c	100±0.00^c

Data are expressed in % inhibition in comparison to that of ascorbic acid standard (mean ±SE of n=3). Values within a group also differ significantly (*P* ≤ 0.05). ^{a}*P*<0.05; ^{b}*P*<0.01 and ^{c}*P*<0.001 significantly more effective as compared to the respective concentration *of G. sylvestre* .

***In vitro* LPO**

Significant increase in LPO (*P*<0.001) was seen in all referred concentrations of $FeSO_4$, whereas the highest increase was found in 10 mM in both the organs (Table 5). A concentration dependent inhibition in LPO was found by all the test drugs. However, in both liver and kidney tissue homogenates, the highest inhibition was observed in Madhuhari, while in case of other drugs the order of antiperoxidative activity was Diabecon> *G. sylvestry*> Madhumehari and the drugs concentrations below 0.1 mg/ml were ineffective. (Table 6 & 7)

Table5: Induction of LPO in liver and kidney tissue homogenates by $FeSO_4$.

Tissue	Control	5 mM	10 mM	15 mM	20 mM
Liver	0.873±0.012	2.03±0.093** (132.86%)	2.85±0.012*** (226.92%)	2.63±0.380* (202.10%)	2.14±0.247* (145.45%)
Kidney	2.22±0.032	4.02±0.196** (81.44%)	4.57±0.080*** (106.05%)	3.83±0.273* (72.51%)	3.79±0.073*** (70.72%)

Data are expressed in mean ±SE (% increase) of three measurements and presented as nM MDA formed /h/mg protein. * *P*<0.01; ** *P*<0.001 and *** *P*<0.0001 significant increase in LPO as compared to the respective control values.

Table 6: Inhibition of LPO by different test drugs (mg/ml of DW) induced by $FeSO_4$ (10 mM in DW) in liver tissue homogenate.

Drugs conc.	Control	$FeSO_4$	$FeSO_4$+0.1mg	$FeSO_4$+1mg	$FeSO_4$+10mg	$FeSO_4$+20mg	$FeSO_4$+30mg
Diabecon	1.93±0.10	7.01±0.01 (262.24%)	4.01±0.42 (42.73%)	3.08±0.38 (56.04%)	1.15±0.06[b] (83.55%)	2.39±0.08[b] (65.85%)	4.25±0.21 (39.42%)
Madhuhari	1.93±0.10	7.01±0.01 (262.24%)	3.76±0.15 (46.35%)	1.04±0.08[b] (85.12%)	2.30±0.12[b] (67.16%)	3.06±0.14[b] (56.34%)	4.07±0.03 (41.91%)
Madhumehari	1.93±0.10	7.01±0.01 (262.24%)	3.37±0.20 (33.31%)	1.92±0.11[a] (51.94%)	2.47±0.08[a] (72.52%)	2.98±0.09[b] (64.67%)	4.67±0.77 (26.12%)
G. sylvestre	1.93±0.10	7.01±0.01 (262.24%)	4.22±0.24 (39.77%)	2.53±0.21 (63.84%)	1.41±0.08 (79.80%)	4.45±0.24 (36.45%)	4.96±0.52 (29.17%)

Data are expressed in mean ±SE of three measurements (% inhibition) and presented as nM MDA formed/ h /mg protein in comparison to that of $FeSO_4$ treated tubes (mean ±SE of three measurements). All drug treated tubes showed significant ($P < 0.001$) inhibition as compared to the values of $FeSO_4$ treated tubes. [a] $P<0.05$ and [b] $P<0.01$ indicate significant inhibition as compared to the respective value of *G. sylvestre* extract at the similar concentration.

Table 7: Inhibition of LPO by different test drugs (mg/ml of DW) induced by $FeSO_4$ (10 mM in DW) in kidney tissue homogenate.

Drugs conc.	Control	$FeSO_4$	$FeSO_4$+1mg	$FeSO_4$+10mg	$FeSO_4$+20mg	$FeSO_4$+30mg	$FeSO_4$+40mg
Diabecon	1.74±0.06	4.62±0.04 (165.37%)	3.22±0.04[b] (30.26%)	2.03±0.08[b] (55.89%)	1.18±0.16[c] (74.30%)	1.01±0.07[b] (77.94%)	2.37±0.30[a] (48.68%)
Madhuhari	1.74±0.06	4.62±0.04 (165.37%)	0.68±0.01[c] (85.23%)	1.54±0.05 (66.62%)	2.10±0.04 (54.43%)	3.22±0.08 (30.19%)	3.73±0.29 (19.20%)
Madhumehari	1.74±0.06	4.62±0.04 (165.37%)	3.70±0.22 (19.81%)	3.04±0.05 (34.17%)	2.90±0.02 (37.21%)	1.74±0.11[b] (62.31%)	2.77±0.15 (39.86%)
G. sylvestre	1.74±0.06	4.62±0.04 (165.37%)	2.59±0.02 (43.84%)	1.42±0.16 (69.07%)	2.42±0.23 (47.54%)	2.98±0.02 (35.36%)	3.78±0.09 (18.41%)

Data are expressed in mean ±SE of three measurements and presented as nM MDA formed/ h /mg protein and % inhibition in LPO in comparison to that of $FeSO_4$ treated tubes (mean ±SE of three measurements). All drug treated tubes showed significant ($P < 0.001$) inhibition as compared to $FeSO_4$ added tubes. [a] $P<0.05$, [b] $P<0.01$, [c] $P<0.001$, significantly more effective as compared to the respective concentration of *G. sylvestre.*

Polyphenols and flavonoid contents

The total phenolics and flavonoids obtained are summarized in table 8. Both the compounds were highest in quantity in Madhuhari and lowest in *G. Sylvestre* extract among all three test drugs.

DISCUSSION

Results of our study revealed that three test polyherbal formulations as well as their common ingredient, *G. sylvestre* exhibit different degrees of antioxidative/ antiperoxidative potential depending on their concentrations and depending on the tissue system used. This study also established that a better efficacy is exhibited by PHFs as compared to the mono-herb preparation.

Although some reports are there on the antioxidative property of the individual plant extract present in these formulations [21, 28], scientific studies on the antioxidative potential of herbal formulations are extremely meager [11]. Particularly, on the comparative aspect of antidiabetic formulations, this report appears to be the first one. In our study, although all three PHFs were found to be anti-peroxidative, the observed highest antioxidative potency of Madhuhari certainly suggests that it is more beneficial as compared to other tested drugs.

As DPPH is a stable free radical and a suitable model compound to analyze antioxidative potential of natural drugs [20], our results on this compound appear to be relevant. Our findings also revealed the presence of direct nitrogen radical scavenging ingredients in the test drugs. Similar pattern in NO assay, reported earlier by Zhou *et al* [29] also supports the present findings. As in *in-vivo* system, oxidative stress via NO radical, severely damages β-cells of pancreas and leads to diabetes mellitus [30, 31], the drugs having better NO inhibition efficacy as observed in the present investigation can be considered as highly effective *in vivo* antioxidant [29,32].

Since reports on comparative study of PHFs of the present or other drugs are negligible, the present findings can be compared with that of other polyherbal drugs [3,33-35] or with mono-herbal drugs [11,20,24,36], which also indicate a better efficacy of PHFs over mono-herbal drug. Our study also reports dose dependent effects of the test drugs. Since the principal ingredient in a drug is very often considered as responsible for its efficacy, we compared the effects of the test PHFs with their main component, *G. Sylvestre*. Interestingly, the antioxidative effects of the former were found to be greater than that of later suggesting the use of PHFs over mono-herb based drug [20].

The ferric and hydroxyl radicals scavenging activity of test drugs was estimated by reducing power assay, because of the fact that both the ions are frequently formed in any living system and their overproduction or their lesser removal may cause several metabolic imbalances [3, 37]. Thus, the possible mode of action of the test drugs could be either their direct hydroxyl radical scavenging action or through chelating ferric/ ferrous ions in the reaction mixture. Interestingly in our study we observed that the test drugs were able to scavenge both the radicals.

For years $FeSO_4$ is known as potent oxidizing agent which forms ferryl-perferryl complex by Fenton reaction and this product leads to oxidative stress. We also found a higher TBARS level in $FeSO_4$ treated tissue homogenates which was consistent with previous findings [20,23,24,26,].

Earlier other hypoglycemic herbs have been evaluated for their *in vitro* antioxidative activity. These include extracts of *Momordica charantia*, *Eugenia jambolana*, *Tinospora cardifolia, Allium sativum, Ocimum sanctum, Phyllanthus amarus, Pterocarpus marsupium, Gymnema sylvestre* and *Withania somnifera* showing strong antioxidative, free radical scavenging and antilipid- peroxidative activities [10,36]. All these plants are also reported to have significant anti-hyperlipidemic and antioxidative properties in *in vivo* [6]. However, nothing significant study was made on hypoglycaemic formulations.

The present findings indicate that plant derived antioxidative activity of the test drugs might have been mediated through an inhibition of hydroxyl radical generation, reduction of total amount of ferrous/ferric ions available for chain reaction and/or metal chelation activities which may block the reaction at any instant. In number of studies it was found that herbal drugs having *in vitro* LPO inhibitory activity promisingly served as antioxidant *in vivo* [8,24]. Sometimes it becomes challenging to extrapolate results from complex *in vivo* systems or from single *in vitro* assay [2,37,38]. Therefore, for more accurate results, different free radical

scavenging assays have been employed from time to time [24,39]. Interestingly we found more or less positive effects with all the test drugs.

A few reports are available on hypoglycaemic and hypolipidemic potential of Diabecon [21], Madhuhari [40] and Madhumehari [42]. However, these did not indicate their mechanism of action. Secondly, PHFs cannot be interpreted by available reports of their individual herbs, because in PHFs there is a possibility of chemical interactions among natural components during processing, preparation and/or administration [33]. Moreover, synergism or antagonism between phytochemicals might exert different action(s) on the same molecular target/receptor or may change the bioavailability of other. In a case study it was found that the individual components of anti-cancer polyherbal drug, Wu-chang-an (WCA), worked on different target molecules and potentiated the effect of each other showing synergism [16]. On the other hand β-sitosterol-D-glucoside showed strong therapeutic activity in pure form but in co-occurrence of other compounds it lost its activity [39]. Similar results were also found with other drugs [15,37,38]. Other than these reasons certain pharmacokinetic situations, phytochemical metabolism, transport rates of molecules and target receptors binding affinity may differ completely than effects of their individual herbs [15-18].

Table 8: Flavonoids, total Polyphenol and EC_{50} values of different test drugs.

Drugs	Total flavonoid	Total polyphenols	NO Scavenging. Assay, EC_{50}	Reducing power assay, EC_{50}	DPPH assay, EC_{50}
Diabecon	107.13 ± 8.16	68.76±2.41	12.11	1.493	122.25
Madhuhari	124.61 ± 8.46	92.66±6.94	11.74	0.469	113.55
Madhumehari	71.21 ± 12.39	50.42±2.96	12.29	0.473	126.21
G. sylvestre	53.07 ± 9.51	44.08±3.96	13.09	1.29	167.96
Ascorbic acid	-	-	10.06	0.045	84.56

Data are expressed in mean ± SE of three measurements. Amount of total flavonoids are presented as mg quercetin equivalent / 100 g dry weight of drug and total polyphenols are presented as mg gallic acid equivalent /100 gm dry weight of drug.

When the total polyphenolics and flavonoids of each test drug were measured, their content justified the free radical scavenging strengths [23,24]. In fact, polyphenols are known for their direct free radical scavenging and oxidation resistance activities [16]. Interestingly, we also observed enhanced LPO in higher concentrations, similar to some other herbal medicines [1,2,14,19,38]. These harmful/ peroxidative effects at higher concentrations could be due to the various polymerization reactions and spatial conformations of polyphenolic and/or flavonoid compounds present in drugs that might have modified the reactivity of molecules [41-44].

CONCLUSION

Our findings clearly reveal that despite all three test drugs exhibiting different degrees of antioxidative /free radical scavenging activities in dose dependant manner, all PHFs show their pro-oxidative nature at higher concentrations. Although some assay specific variations on the efficacy of drugs were found, all the drugs were more antioxidative in nature as compared to the mono-herb preparation, *G. Sylvester* extract. Towards the mode of action(s), effective antioxidative potential of the PHFs appears to be dependent on their phytochemicals such as polyphenolics and flavonoid.

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