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ANTIOXIDANT POTENTIAL OF *EUGENIAJAMBOLANA SEED*; A RANDOMIZED CLINICAL TRIAL IN TYPE 2DIABETES MELLITUS.

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ABSTRACT

Diabetes mellitus (DM) is a growing global problem where, increased oxygen free radicals are responsible for the complications. Clinical evidence of *Eugenia jambolana* seeds for use in DM is lacking. This is an open labeled, randomized trial in DM patients: fifteen in group1 received *Eugenia Jambolana* seed powder 10 gm orally per day along with diet and exercise, and ten in group 2 on diet and exercise alone. Follow-up visits were: V_0=randomization; V_1-V_6, after one to six months of therapy. Patients were followed up for blood sugar, glycosylated hemoglobin, fasting insulin, antioxidants from V_0 to V_6 in group 1 and V_0 to V_3 in group 2. Results revealed in group1: reduced Fasting blood sugar (FBS) fromV_3-V_6 [140(18), 125(26.5), 124(19.5), 134(24)]; rise in SOD (superoxide dismutase) at V_3[3255(2394)] and V_6[4650(3517)]; reduced HOMA-IR (homeostasis model assessment for insulin resistance) at V_3 [5(6.8)] and V_6 [5.5(3.3)]; negative correlation between SOD and FBS at V_3(ρ= -0.5) and V_6(ρ=-0.577) which were significant at ($P<0.05$). Study shows the antihyperglycemic and antioxidant potential of *Eugenia jambolana* seed powder and a negative correlation between the antioxidant and antihyperglycemic action.

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KEYWORDS

Eugenia jambolana seed; Antihyperglycemic action; Antioxidant potential; Insulin resistance.

INTRODUCTION

Diabetes is a global problem with devastating human, social and economic impacts. It is a growing epidemic threat to global healthcare services. Each year more than 3.8 million people die from diabetes related causes, one death every 10 seconds[1]. Chronic complications in diabetes mellitus (DM); affects many organ systems and is responsible for the morbidity and mortality in this disease. Reactive oxygen species or superoxide in the mitochondria resulting from hyperglycemia has been proposed as a possible unifying mechanism that activates the four possible molecular mechanisms of increased: advanced glycation end products; sorbitol; diacylglycerol; and fructose 6 phosphate to explain these chronic complications[2]. Hence, use of an antioxidant with antihyperglycemics in treatment of DM is justifiable. Although, antioxidant studies in experimental models and observational studies suggest strongly that antioxidant should confer beneficial effects in reducing complications in diabetes, clinical evidence for their benefits in DM is lacking. Clinical trials with conventional antioxidants in diabetics are limited and inconclusive except for the α-lipoic acid study in diabetic neuropathy[3]. Hence, there is a compelling need for more clinical research on this topic.

Traditional drugs have given leads in drug search resulting in the discovery of novel molecules. *Eugenia jambolana (EJ)* an indigenous Indian medicinal plant has been found useful in therapy of DM [4, 5]. Animal studies have shown the antihyperglycemic and antioxidant activities of *EJ* seed powder [6,7].

The aim of this study was to assess the antioxidant potential of Madhuharachurna [Abhinava Vidyatheertha Ayurveda (AVA) Trust Regd] a product of *Eugenia jambolana* dried seed powder in therapy of newly detected (mild to moderate) type 2 DM patients by estimating antioxidants in erythrocytes and correlating the antidiabetic activity of *Eugenia jambolana* with its potential antioxidant activity.

MATERIALS AND METHODS

Patients

Newly detected diabetes patients attending the medicine outpatient at Kasturba Medical College hospital (KMC), Mangalore between July 2006-August 2007 were enrolled. Inclusion criteria were: men and women ≥ 35 years; newly detected type 2 DM not on antidiabetic therapy, glycosylated hemoglobin (HbA_1c) between 6% and 9%, fasting blood sugar (FBS) ≥126mg/dl (7mmol/dl) and ≤ 200mg/dl (11mmol/dl); values of hemoglobin and serum creatinine within 20% of lower/upper limits respectively of the normal range for the reference laboratory, liver enzymes within; the normal range or 30% above the upper limits of normal. Exclusion criteria were: type 2 DM patients on therapy and history of diabetic complications; presence of: acute / uncontrollable illnesses; acute ischemia, arrhythmia, stroke in the past six months, BP>180/150; on complementary therapies (ayurvedic/homeopathic/herbal); women of childbearing age.

Study drug

Madhuharachurna [Abhinava Vidyatheertha Ayurveda (AVA) Trust Regd], a product of *Eugenia jambolana (EJ)* dried seed powder required for the study was obtained from Srimad Abhinava Vidyatheertha Ayurveda [AVA] Trust Dakshina Kannada district, Karnataka, India. The *EJ* seeds used for

conducting the trial were collected in the month of April-May from trees grown in Dakshina Kannada district, dried in sunlight and the seed powder was prepared by [AVA Trust], an Ayurvedic establishment. Specimens of the collected material used were matched with authenticated voucher specimens; numbers 124678,124679 identified by Dr. Kakunje Gopalkrishna Bhat and deposited in the Herbarium of the Botanical Survey of India (BSI), Pune on 13th July 2004. Four batches of Madhuhara churna [AVA Trust Regd] were eluted for the percentages of ellagic acid and gallic acid. Each batch of the seed powder was standardized at the department of Pharmacognosy, Manipal college of Pharmaceutical Sciences, Manipal before dispensing. The HPTLC analysis report on percentages (%) of ellagic acid (weight for weight=w/w) were 0.17; 2.772; 1.42; 1.94 and of gallic acid(weight for weight) were 0.33 ; 4.42 ; 4.39 ; 4.11. A 5 gm sachet was made, after weighing in an electronic balance.

Experimental design

This was an open labeled, randomized study. Newly diagnosed patients with type 2 DM were counseled (verbal and written) on the importance of a diabetic diet, calorie restriction up to of 1500 kcal/day and exercise (brisk walking for 45 minutes /day) in the lifestyle modification clinic of KMC hospital, for 4 weeks (screening period). Patients whose FBS at the end of the screening period fell within the WHO diagnostic criteria for DM >7mmol/dl and <11 mmol/dl were enrolled for the study. A total of 25 patients were randomized based on computer generated randomization list into the following groups: group 1 (n=15), were given the test drug *EJ* seed powder 5g at twelve hourly intervals; group 2 (n=10) were on diet and calorie restriction with exercise therapy (not on any medication). As all inclusion criteria were followed and it was not feasible to extend the duration of enrollment of the patients for technical reasons, numbers of patients in groups 2 remained at 10. A compliance card was given to the patient for registration of the following: daily drug intake; adherence to diet as instructed by the dietician; nature and duration of exercise. The compliance card was brought by the patient at follow-up visits with unused drugs (if any). Compliance was verified at each visit with this card and by responses to telephone calls which were made once in 15 days to register the importance of regular drug intake, dietary restrictions and exercise. Compliance was also monitored by recording the body weight, waist: hip ratio and body mass index (BMI) kg/m^2 using standardized equipment. The follow-up visits were labeled: V_0= Randomization; V_1, V_2, V_3, V_4, V_5, V_6, after 1, 2, 3, 4, 5, 6 months of therapy. Diet, calorie restriction and exercise were continued in both groups during the study period. The duration of the study was: six months for group 1; and three months for group 2 as advised by the Institutional Ethics Committee (IEC) as these patients were not on any medication.

Patients were followed up for the following values: fasting blood glucose (FBS) and postprandial plasma glucose (PPBS) at V_0 and monthly from visits V_1- V_6 in group 1 and visits V_1 –V_3 in group 2; glycosylated hemoglobin (HbA_1c), fasting insulin, antioxidants [viz., reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT)] at visits V_0, V_3, V_6 in group 1 and V_0, V_3 in group 2.

The study was conducted in compliance with 'Ethical Guidelines for Biomedical Research on human subjects Indian council of medical research (ICMR) 2000'-- conduct of trial with herbal remedies and International conference on harmonization (ICH)/Good Clinical Practice (GCP) guidelines. Study was conducted after institutional ethics committee approval and subjects were enrolled after obtaining informed consent.

Methodology

The plasma glucose was estimated by GOD-POD method by a fully automated analyzer (Hitachi 917 Rack model) using kits

from Aspen Laboratories Private limited, India. The method was based on the formation of red quinine by combination of 4-aminoantipyrine with nascent oxygen released by the action of glucose oxidase and peroxidase enzymes. The intensity of red colour is measured at 505nm using spectrophotometer [8]. The reduced glutathione (GSH) content of the erythrocytes was determined by the method of Beutler et al.[9]. The method is based on the development of a relatively stable yellow color when 5, 5 dithiobis 2-nitrobenzoic acid (DTNB) is added to sulphydryl compounds. The intensity of the color is measured at 412nm using spectrophotometer. Superoxide dismutase was assayed by McCord and Fridovich method [10]. Illumination of riboflavin in the presence of oxygen and electron donor like methionine or ethylenediamine tetra acetic acid (EDTA) generates superoxide anions. Reduction of nitrobluetetrazolium (NBT) by superoxide was followed at 560nm using spectrophotometer. The catalase activity of the hemolysate was determined by adopting Brannan et al. method [11]. The assay is based on the disappearance of hydrogen peroxide in the presence of the enzyme source at 26°C.The intensity of the color was measured at 505nm using spectrophotometer. Hemoglobin was estimated by cyanomethemoglobin method by Varley H et al. which is based on the formation of cyanomethemoglobin by ferricyanide and cyanide[12]. The intensity of the color developed is measured at 540nm using spectrophotometer.

STATISTICAL ANALYSES

Statistical difference between the groups were assessed by Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables. Wilcoxon signed rank test was applied for within group analysis. Spearman's Rank Correlation Coefficient (ρ) was applied for correlation of SOD with FBS. Data was analyzed by SPSS versions 14. *P-value<0.05* was considered statistically significant.

RESULTS

Eugenia jambolana treated group1 did not differ from control group 2 in terms of baseline characteristics age, gender, BMI, hematological, blood biochemistry or antioxidant values

Table 1
Baseline characteristics of group 1 and group 2

	Group1 (n=15)	Group 2 (n=10)	P
Age (yr)	54(49-62)	54(41-63)	0.723
Sex (M/F)	11/4	6/4	0.668
BMI (Kg/m^2)	24.58(21.78-27.73)	25(23.72-27.25)	0.428
FBS (mg/dL)	150(135-171)	140(117-153)	0.16
PPBS (mg/dL)	197(161-247)	187.5(148-226)	0.522
HbA1c (%)	7.9(7-9)	7.4(6.9-8.6)	0.461
Insulin (µU/ml)	26.05(14-36)	19.35(14-66)	0.879
HOMA IR	11.2(6-14)	6.8(4-20)	0.709
GSH (µmol)	4.13(2.4-5.9)	4.67(2-9)	0.849
SOD (U/gm Hb)	1367.5(859-2958)	1663.5(1567-2721)	0.765
CAT (U/gm Hb)	21725(13572-25551)	20989.85(3629-54200)	0.815

Values are expressed as median (IQR); age as male to female ratio. Mann-whitney U (2 tailed) test applied for continuous variables and Fisher's exact test for categorical variables. P<0.05 is considered significant

In group 1, the test drug *Eugenia jambolana* seed powder (*EJ*) reduced FBS at V_3, V_4, V_5, V_6 compared to baseline values at V_0 and there was no significant reduction in FBS values in group 2. No reduction in PPBS and HbA_1c values were observed in both groups (Table 2).

Table 2
Median values in FBS, PPBS, HbA_1c according to treatment groups, V_0 -V_6

Group 1	FBS(mg/dL)	PPBS(mg/dL)	HbA1c (%)
V0	150(36)	197(86.5)	7.9(2)
V1	140(32)	210(95.5)	
V2	140(33)	221(89)	
V3	140(18)*	197(68)	7.9(1.88)
V4	125(26.5)*	202(68)	
V5	124(19.5)*	171(62.5)	
V6	134(24)*	184(85.5)	7.4(1.05)
Group 2			
V0	140(36)	187.5(78)	7.4(1.6)
V1	132(36)	142(96.5)	
V2	123.5(21)	144.5(26)	
V3	127.5(17.5)	148.5(126)	7.25(1.5)

*Values are expressed as Median (Interquartile range in parenthesis); V_0= Randomization; V_1, V_2, V_3, V_4, V_5, V_6, after 1, 2, 3, 4, 5, 6 months of therapy. Wilcoxon sign rank test applied for analysis. * p<0.05 considered significant*

Homeostatic model assessment for insulin resistance (HOMA-IR) indicates insulin resistance, and is obtained by FBS mmol/L x fasting insulin µU/ml÷22. A value > 6.8 is considered insulin resistant. In group1 alone, there was significant reduction in HOMA-IR at V_3 and V_6 (Fig 1).There was no significant changes in fasting insulin levels in both the groups

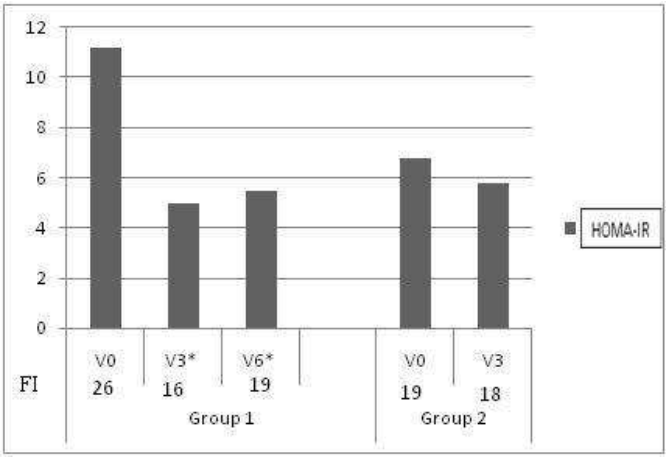

Figure1
Bar graph representing median values of HOMA-IR at V_0, V_3, V_6. V_0, V_3, V_6 represents visits randomization, three months and six months after therapy respectively according to treatment groups. HOMA-IR=homeostasis model assessment insulin resistance. Wilcoxon sign rank test applied for analysis. FI=fasting insulin levels expressed as µU/ml; * p <0.05 significant

Among endogenous antioxidants superoxide dismutase (SOD) alone increased significantly at V_3 and V_6 in group 1 (Table 3).

Table 3
Median values of GSH, SOD, CAT at V_0, V_3, V_6 according to treatment groups

	Visits	Group 1	Group 2
	V0	4 (3.5)	4.7 (6.8)
GSH	V3	3.8 (6.2)	4.3 (6.8)
µmol	V6	4.5 (2.6)	
	V0	1367.5 (2099)	1663.5 (1154)
SOD	V3	3255.4* (2394)	1461.9 (1400)
U/gm Hb	V6	4650* (3517)	
	V0	21725 (11979)	20990 (50571)
CAT	V3	15966.8 (5786)	19879 (27034)
U/gm Hb	V6	15578 (14830)	

*Values expressed as median (IQR); V_0,V_3,V_6 represents visits randomization, three months and six months after therapy respectively. Wilcoxon sign rank test was applied for analysis. * $p<0.05$ is considered significant*

In group 1 at V_3 and V_6 there was negative correlation: between rise in SOD value and decrease in FBS value (Table 4).

Table 4
Correlation of SOD with FBS in group 1

Visit	V_3	V_6
FBS	–0.5*	-0.577*

*Spearman's Rank Correlation Coefficient (ρ) was applied.V_3,V_6=at the end of three and six months after therapy respectively. *p <0.05 significant*

There was no significant change in waist: hip ratio, safety parameters, compliance to drug, compliance to diet restriction and exercise in both groups from base line to end of the study

DISCUSSION

The present study demonstrates the antihyperglycemic effect of Madhuharachurna [AVA Trust Regd] in type 2 diabetic patients wherein, there was significant reduction in FBS values with negligible changes in PPBS values during the study period in group 1. Animal studies have shown that *EJ* seed powder exhibits pancreatic action to reduce blood sugar [7]. Studies by Bansal et al.[13] have shown the ability of *EJ* powder to enhance the conversion of proinsulin to insulin. Significant reduction observed only in FBS, maintenance of PPBS values at the baseline level throughout the study period and no increase in serum insulin levels at any visits suggest that *EJ* seed powder also exhibits an extrapancreatic action, besides the pancreatic action as suggested by previous studies[7]. Many animal studies have shown its extrapancreatic action. This extrapancreatic action could be due to its

action on the liver to decrease blood glucose levels by enhancing glycogenesis[6] or by restoring carbohydrate enzyme activity [14]. Stronger evidence on the extra pancreatic mechanism of action of *EJ* would have come from a decrease in postmeal serum insulin levels which however was not assayed in this study. Surprisingly there is no change in PPBS values from baseline. It may be a result of prolonged latency of the drug to initiate post meal insulin secretion from β islet cells in the pancreas or that the mechanism of action of *EJ* leans forwards towards important extra pancreatic dynamics that do not relate early enough for control of PPBS. The delayed effects on FBS (three months of therapy), maintaining PPBS and HbA1c (Table 2) at the same level as that of the baseline throughout the study implies the dosage used was less than the optimum and titrating the dose and increasing the frequency of administration (three times daily) will be more effective, as suggested by Helmstädter A that a higher dose is required for a clinical trial using EJ seeds[15].

Though the individual contribution and/or integrated role of FBS and PPBS on glycemic control (HbA_1c) remains a subject of debate[16], findings in our study with reference to group 1 treated with *EJ* seed powder: sustained, significant reductions in FBS values from V_0-V_6 ; and a clinical impression of a reduction in HbA_1c values from V_0-V_6 (7.9-7.4), which however did not hold the tests of statistics is supportive of the conclusion of Louis Monnier et al.[17] which states that when basal HbA_1c values is >7.5, basal hyperglycemia/FBS plays a predominant role on glycemic control as compared to the proposed role of PPBS on HbA_{1C} .

A significant finding of our study was increase in SOD value at V_3 and V_6 in group 1 (Table 3). The changes in the values of GSH and CAT in group 1 were not statistically significant. Studies have shown antioxidant effect of EJ by increasing SOD level[18]. The rise in SOD could be due to direct enzyme induction by *EJ* seed powder as work done on plant products have reported their propensity to induce antioxidants[19,20]. The ability of *EJ* seed powder to enhance SOD values is further supported in this study by the findings in group 2 where despite a reduction in FBS levels by diet and exercise therapy alone there was a substantial fall in SOD values.

Pathophysiological concentrations of glucose are known to promote oxidative modification of LDL by superoxide-dependent pathway as evidenced by an increase in superoxide and H_2O_2 that results in the death of the β islets cells[21,22]. This finding implies that SOD, the enzyme that quenches superoxides plays an important role in DM both: by preventing death of islet β cells and the eventual progress of DM and; by reducing the levels of oxidative radicals which are known to play a permissive role in the chronic complications of DM. Studies have shown *EJ* seeds by enhancing antioxidant enzymes protects beta cells in pancreas[23].The ability of *EJ* to enhance SOD levels therefore, adds concurrence to the extrapancreatic action of *EJ* seed powder in the chronic treatment of DM. This proposition justifies the addition of *EJ* seed powder to established antihyperglycemic treatment modalities in the long-term management of DM.

Insulin resistance an important mechanism in the development and progress of DM and atherosclerosis is indexed by HOMA-IR (homeostasis model assessment for insulin resistance)[24]. In the present study, at V_3 and V_6 there was a significant fall in HOMA-IR (index of insulin resistance) in group 1 (Figure 1). The negative correlation between rise in SOD level and reduction in FBS values at mid study and end of study (Table 4), as well as reduction in insulin resistance / HOMA-IR at midstudy and end of study implicates a robust association between the antioxidant and antidiabetic action of *EJ* seed powder described in this study.

Results of this study validate the antioxidant potential and antihyperglycemic action of Madhuharachurna [AVA Trust Regd]

a product of *EJ* seed powder and its usefulness as add- on to established treatment modalities in the management of DM preserving thus, ethnic beliefs in large populations, which is an important goal in developing countries.

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