January 2017

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Studies on antihyperlipidemic and endothelium modulatory activities of polyherbal formulation (POL⁴) and its ingredients in high fat diet-fed rats

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Abstract: A compound herbal formulation (POL⁴) is used traditionally in interior parts (Distt. Badin) of Sindh, Pakistan, for the treatment of metabolic disorders like diabetes and hyperlipidemia. This study is aimed to determine the effectiveness of POL⁴ and its ingredients in hyperlipidemia and associated endothelial dysfunction and hypertension. POL⁴ is composed of equal proportion of Nigella sativa, Cichorium intybus, Trigonella foenum graecum and Gymnema sylvestre mixed in powdered form. Chronic (6 to 7 weeks) administration of POL⁴ and its ingredients mixed in diet caused a notable attenuation in total cholesterol, low density lipoprotein cholesterol, triglycerides, atherogenic index, C-reactive protein and glucose, while it has increased high density lipoprotein levels. POL⁴ intervention markedly (p<0.01) reduced systolic blood pressure in rats to 127±1.92 vs. 145.4±1.07 mm of Hg using tail-cuff method and significantly (p<0.05) improved endothelium-dependent relaxation (75±2.88 vs. 82.75±1.22%) to acetylcholine in isolated aortae of rats in treatment groups using force transducer and PowerLab system. Similar activities were assessed on the part of ingredients of POL⁴. These findings indicate that POL⁴ and its ingredients possess antihyperlipidemic, endothelium-dependent modulatory and antihypertensive activities, thus providing an evidence to the vernacular use of POL⁴ in hyperlipidemia and hypertension.

Keywords: Poly herbal formulation, antihyperlipidemic, antihypertensive, endothelial modulatory.

INTRODUCTION

Persistent hyperlipidemia is a major contributing factor precipitating cardiovascular disorders (CVDs) such as, hypertension, myocardial infarction, peripheral artery and coronary artery diseases (Orsó et al., 2009). Hyperlipidemia expedites the process of atherosclerosis and its consequences of developing multi-vessels disease, heart failure and stroke. Controlled lipid levels achieved by non-pharmacological or pharmacological interventions are known to protect from cardiovascular events (Scicchitano et al., 2014). It is well documented that pre-hyperlipidemia is highly prevalent in developing (around 80%) countries including Pakistan compared to the developed countries (Dodani et al., 2008), which might be associated with sedentary life style and unhealthy dietary habits. The available treatment options for hyperlipidemia include statins, fibrates, bile acid sequestrants and niacin, however, their prolonged use is associated with multiple adverse effects like rhabdomyolysis, cardiomyopathy, myopathy, osteoporosis, constipation, intolerance and poor compliance (Last et al., 2011; Casula et al., 2012). In Asian countries including Pakistan, the alternative approaches, especially the use of herbal remedies and supplements are becoming very popular nowadays for their medical uses (Kajal et al., 2016). Herbal remedies have gained maximum attention in current era because of the presence of millions of constituents offering diverse range of activities with inbuilt potential to nullify associated adverse effects (Salvamani et al., 2014). One of the oldest traditional systems of the world, Greeco-Arab/Tibb-e-Unani medicine is commonly practiced in South Asian countries. In our settings due to cultural acceptance, people prefer using medicinal plants or herbal formulations for various chronic ailments like constipation and diarrhea (Mehmood et al., 2015), hypercholesterolemia (Naz et al., 2016), hypertension and vascular resistance (Aziz et al., 2013; 2009). Similarly, formulations like a combination of S. reticulate, C. zeylanicum, L. speciosa, C. sinensis and G. sylvester for diabetes (Baig et al., 2014), Angiosifa(Parasuraman et al., 2013) and Itrifal Saghir (Kamali et al., 2012) are popular for their therapeutic use as antihyperlipidemic and anti-obesity, respectively. A compound herbal formulation (POL⁴) is popular in our system of traditional medicine for its medicinal utility in cardiometabolic disorders (table 1), however no report is available in the literature to rationalize its medicinal uses in hyperlipidemia and hypertension.

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This study is aimed to validate the medicinal use of the parent formulation and its ingredients in hyperlipidemia, endothelial dysfunction and hypertension using high fat diet-induced hyperlipidemic rat model.

MATERIALS AND METHODS

Identification and preparation of polyherbal (POL4) formulation

The ingredients [N. sativa (Ns), C. intybus (Ci), T. foenum graecum (Tfg) and G. sylvestre (Gs)] of polyherbal (POL4) formulation were procured from Rehmania Pinsar Store, Sargodha and samples were identified by Dr. Muhammad Amin Ullah Shah, Taxonomist, Department of Botany, University of Sargodha, Sargodha, Pakistan. The individual samples were deposited in the faculty herbarium with following voucher numbers; N. sativa (Malik-632), C. intybus (Malik-633), T. foenum graecum (Malik-634) and G. sylvestre (Malik-635). All the ingredients were powdered and mixed in equal parts to compose POL4. For further studies, the parent formulation and its ingredients were mixed in the diet of different HFD-fed animal groups.

Animals

Sprague-Dawley rats (180-250g) of either sex, kept at the “Animal House” of Aga Khan University, Karachi, were used in this study. The animals were maintained at moderate temperature (23±5°C), acceptable relative humidity (55±5%), 12-hr light/dark periods and were kept in plastic cages containing sawdust (changed after two days). Animals were given normal diet and tap water. All the animals were grouped at random in desired number of groups by numbering method and were acclimatized for 5 to 7 days before starting the experiment. The experimental protocol were approved from institutional ethics committee at University of Sargodha, Sargodha, Pakistan.

Chemicals

Cholesterol, cholic acid, atorvastatin, acetylcholine and phenylephrine were sourced from Sigma Chemicals, St. Louis, MO, USA. Isoflurane was obtained from the Pharmacy of Aga Khan University Hospital, Karachi, Pakistan. Butterfat was procured from United King Bakers, Bahadurabad, Karachi, Pakistan. The reagents for the assessment of serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein-cholesterol, triglycerides, glucose and C-reactive protein levels were purchased from Roche Diagnostic Karachi, Pakistan, while their estimation was carried out using Cobas c111 autoanalyzer diagnostic system (Roche Diagnostics International Ltd., Switzerland).

Preparation of different diets and intervention

Different diets were used in this study. Normal diet (ND) was developed at the animal house of Aga Khan University. ND consists of (gram/kilogram): fiber 380, flour 380, molasses 12, powdered milk 150, sodium chloride 5.8, vegetable oil 38, potassium metabisulphate 1.2, nutrivet-L 2.5, fish meal 170. A total of 56 rats were randomly divided into 8 groups. Animals in group 1 (n=7) were administered the normal diet. High fat diet (HFD) was prepared using combination of cholesterol, cholic acid and butter fat (2, 0.5 and 5% w/w), respectively, an addition to normal diet components for 6-7 weeks (Aziz et al., 2013). Animals in groups 2-8 (n=7 in each group) were administered HFD. The animals in groups 4-8 were

Table 1: Showing description of the ingredients of compound herbal formulation

<table>
<thead>
<tr>
<th>Ingredients of POL4 (Botanicals)</th>
<th>Vernacular names/part used</th>
<th>Family</th>
<th>Medicinal uses</th>
<th>Phytochemical Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigella sativa, L.</td>
<td>Black seed, Kalonji /seeds</td>
<td>Ranunculaceae</td>
<td>Hypcholesterolemic, hypoglycemic, cardiodepressant (Razavi and Hosseinzadeh, 2014)</td>
<td>Flavonoids, saponins, thymoquinone and tocopherols (Khan and Afzal, 2016)</td>
</tr>
<tr>
<td>Cichorium intybus, L.</td>
<td>Chicory, Kasni /seeds</td>
<td>Asteraceae</td>
<td>Hypcholesterolemic, hypoglycemic, cardiodepressant, cardiotonic (Nadkarni, 1986; Street et al., 2013)</td>
<td>Tannins, flavonoids, saponins and caffeoylquinic acid (Street et al., 2013)</td>
</tr>
<tr>
<td>Trigonella foenum graecum, L.</td>
<td>Fenugreek, Methi /seeds</td>
<td>Fabaceae</td>
<td>Hypcholesterolemic, antidiabetic, hypotriglyceridemic (Duke et al., 2002; Chaturvedi et al., 2013).</td>
<td>Flavonoids, diosgenin, trigonelline and 4-hydroxyisoleucine (Khorshidian et al., 2016)</td>
</tr>
<tr>
<td>Gymnema sylvestre, R.Br.</td>
<td>Gurmar booti / leaves</td>
<td>Asclepiadaceae</td>
<td>Hypcholesterolemic, cardiotonic, hypoglycemic, antiobesity (Nadkarni, 1986; Tiwari et al., 2014).</td>
<td>Flavonoids, saponins, tannins, gymnemic acid and deacglycennemic acid (Tiwari et al., 2014)</td>
</tr>
</tbody>
</table>
administered POL4 and its ingredients mixed in diet at a dose of 6% w/w, respectively. The animals in group 3 were administered atorvastatin (a standard lipid lowering drug), at 10mg/kg/day dissolved in distilled water and given through oral gavage (Belagali et al., 2013). All measures were ensured for uniform mixing of POL4 and its ingredients with normal diet.

Assessment of non-invasive blood pressure in non-anaesthetized rats
Non-invasive blood pressure was recorded at day 0 and at week 6 and/or 7 of the study using tail cuff plethysmography (Model 92, IITC Inc., Woodland Hills, USA) joined with PowerLab (4/25) data attainment system linked to a computer with installed software of Chart 5.3 (AD Instruments, Sydney, Australia). Prior to the study protocol, the animals were given training skills for easy blood pressure measurement. As soon as the animals got acclimatized for these procedures, 4–6 measurements, within time interval of 4-8 min, of systolic blood pressure in non-anaesthetized rats were attained and the mean values were calculated accordingly. Possible experimental variables like body temperature at 27°C, respiration, body motion and noise intensity were made minimum to get quality results (Aziz et al., 2009).

Measurement of endothelium-dependent vasorelaxation in rat aortic preparations
At terminal day of the experimental protocol, rats fasted for 12-16 hr were euthanized followed by anesthesia with isoflurane (2-5% v/w) by inhalation in a closed chamber until achievement of deep anesthesia. Dissection was performed at earliest to remove thoracic aortae. All the aortae were shifted to Kreb's solution with ingredients in mmol/L: [NaCl (118.4), KCl (4.7), KH₂PO₄ (1.2), MgSO₄ (1.20), NaHCO₃ (25), glucose (11) and CaCl₂ (2.5) with pH 7.4], cleaned of attached fatty tissues and were sliced into rings of 2 to 3 mm length. Afterward aortic rings were stabilized for 45 min at normal tension until achievement of deep anesthesia. Dissection was performed at earliest to remove thoracic aortae. All the aortae were shifted to Kreb's solution with ingredients in mmol/L: [NaCl (118.4), KCl (4.7), KH₂PO₄ (1.2), MgSO₄ (1.20), NaHCO₃ (25), glucose (11) and CaCl₂ (2.5) with pH 7.4], cleaned of attached fatty tissues and were sliced into rings of 2 to 3 mm length. Afterward aortic rings were immersed in tissue bath assembly loading Kreb's solution at 37°C, bubbled with carbogen (95% O₂ and 5% CO₂), connected with a force transducer (50-7905, Harvard Apparatus, USA) and PowerLab data recording system (Model ML845, AD Instruments, Australia). The aortic rings were stabilized for 45 min at normal tension of 2g, with refreshing physiological salt solution at every 15 min. After stabilization, acetylcholine concentration response curves (ACH; 1×10⁻⁸ to 10⁻⁴mol/L) were constructed on phenylephrine (P.E; 1×10⁻⁸mol/L)-induced contractions (Amin et al., 2015).

Biochemical estimations
Estimation of lipid profile, glucose and CRP
The rats were exposed to isoflurane (2-5% v/w by inhalation) in a closed chamber until fully anesthetized. The blood samples were withdrawn in vacutainer by cardiac puncture from overnight fasted rats. The blood samples were centrifuged at 3000 rpm for 10min (Beckman Coulter Allegra X-22, USA) to extract serum. The estimation of serum total cholesterol (TC), low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol (HDL-C), triglycerides, glucose and C-reactive proteins was carried out using Cobas c111 auto analyzer. Body weights were also measured for the determination of weight variations. Atherogenic index (AI) and coronary risk index (CRI) were calculated using formula: AI= TC-HDL-C/HDL-C and CRI= TC/HDL-C.

STATISTICAL ANALYSIS
The data was presented as means ± S.E.M. Student's t-test was applied for comparison between two groups, while One-way ANOVA (analysis of variance) was applied to compare means in multiple groups by Dunnett's and/or Tukey's post-test. P value of less than 0.05 was accounted significantly different. All graphs were constructed using GraphPad Prism software (GraphPad Software, California, USA).

RESULTS
Effects of treatment on serum parameters in high fat diet-induced hyperlipidemic rats
Chronic administration of high fat diet (HFD) caused a significantly increase in serum total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), triglycerides (TG), C-reactive proteins (CRP), atherogenic index (AI) and coronary risk index (CRI), while it has decreased the high density lipoprotein-cholesterol (HDL-C) compared to normal diet control. Administration of POL4 and its ingredients to HFD-fed rats at a dose of 6% w/w reversed the raised parameters including TC, LDL-C, TG, AI, CRI and CRP, while this intervention has also cause a significantly rise in HDL-C levels compared to HFD-fed rats without any treatment. Similar effects have been observed on the part of positive control of atorvastatin (10 mg/kg/day, oral gavage), a standard lipid lowering agent. The body weight was decreased due to slight reduction in daily feed intake in treatment groups compared to control. The comparative effect of different groups on selected serum parameters has been presented in table 2.

Effect of different treatments on systolic blood pressure
The chronic administration of POL4 and its ingredients to high fat diet (HFD)-fed rats significantly reduced systolic blood pressure to 127±1.92 vs. 145.4±1.07mm of Hg (mean ± SEM; n=6–7). The comparative systolic blood pressure lowering effect in different groups has been presented in fig. 1.

Effect of different treatments on endothelium-dependent reactivity
Administration of POL4 and its ingredients to HFD-fed rats caused significant improvement in endothelium-dependent relaxation by augmenting acetylcholine (ACh)-mediated relaxation to 75.0±2.88 vs. 82.75±1.22% (n=6–7). The HFD administration to rats resulted in


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Table 2: Effect of different treatments on serum parameters of HFD-fed rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>HFD</th>
<th>HFD + Atorva</th>
<th>HFD + POL4</th>
<th>HFD + N. sativa</th>
<th>HFD + C. intybus</th>
<th>HFD + T. foenum graecum</th>
<th>HFD + G. sylvester</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>73.5 ± 7.75</td>
<td>317.2 ± 11.82**</td>
<td>143.8 ± 5.29***</td>
<td>169.2 ± 15.47**</td>
<td>164.4 ± 11.05**</td>
<td>156.5 ± 8.42***</td>
<td>175.4 ± 6.63***</td>
<td>177.0 ± 8.00***</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>38.7 ± 4.66</td>
<td>267.6 ± 8.47###</td>
<td>97.8 ± 5.25***</td>
<td>121.2 ± 10.05**</td>
<td>133.6 ± 9.46**</td>
<td>105.8 ± 6.72***</td>
<td>145.2 ± 7.33***</td>
<td>164.8 ± 9.12***</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>32.2 ± 1.32</td>
<td>20.2 ± 1.45**</td>
<td>25.2 ± 1.26%</td>
<td>28.4 ± 3.65**</td>
<td>27.9 ± 2.05**</td>
<td>26.9 ± 1.24*</td>
<td>27.2 ± 1.15*</td>
<td>26.1 ± 3.94*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>64.7 ± 4.95</td>
<td>83.5 ± 5.66***</td>
<td>75.2 ± 1.25*</td>
<td>74.8 ± 3.76**</td>
<td>75.7 ± 3.52*</td>
<td>76.4 ± 2.45*</td>
<td>74.3 ± 3.58*</td>
<td>75.9 ± 5.90*</td>
</tr>
<tr>
<td>Al</td>
<td>1.35 ± 0.17</td>
<td>14.66 ± 3.35***</td>
<td>4.72 ± 1.40**</td>
<td>4.94 ± 1.29**</td>
<td>4.80 ± 0.96**</td>
<td>5.0 ± 1.63**</td>
<td>5.23 ± 1.46**</td>
<td>6.73 ± 0.95*</td>
</tr>
<tr>
<td>CRI</td>
<td>2.3 ± 0.36</td>
<td>15.7 ± 2.02###</td>
<td>5.6 ± 1.14***</td>
<td>5.7 ± 0.95***</td>
<td>5.8 ± 1.07***</td>
<td>6.1 ± 0.75***</td>
<td>6.3 ± 1.02***</td>
<td>5.1 ± 1.24***</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>109.7 ± 5.62</td>
<td>119.4 ± 4.17###</td>
<td>112.2 ± 2.12%</td>
<td>81.2 ± 4.64**</td>
<td>83.6 ± 5.53***</td>
<td>86.8 ± 7.12***</td>
<td>73 ± 4.34***</td>
<td>62.2 ± 5.15***</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.026 ± 0.016</td>
<td>0.153 ± 0.033###</td>
<td>0.147 ± 0.023%</td>
<td>0.144 ± 0.034*</td>
<td>0.145 ± 0.092*</td>
<td>0.146 ± 0.043%</td>
<td>0.144 ± 0.052*</td>
<td>0.147 ± 0.062%</td>
</tr>
<tr>
<td>Weight variation (%</td>
<td>32.2 ± 1.98</td>
<td>47.8 ± 2.22###</td>
<td>40.2 ± 1.05*</td>
<td>37.2 ± 1.56**</td>
<td>36.4 ± 2.33*</td>
<td>39.2 ± 1.46*</td>
<td>37.7 ± 1.68**</td>
<td>38.4 ± 2.11**</td>
</tr>
</tbody>
</table>

Values shown are means ± SEM, n=7, ***p<0.001 and #p<0.01 compared to ND (unpaired student’s t-test), while ns (non-significant), *p<0.05, **p<0.01 and ***p<0.001 compared to HFD (One way-ANOVA followed by Tukey’s post-test).

POL4 (poly herbal formulation); Ns (N. sativa); Ci (C. intybus); Tfg (T. foenum graecum); Gs (G. sylvester); ND (normal diet); HFD (high fat diet); TC (total cholesterol); LDL-C (low density lipoprotein-cholesterol); HDL-C (high density lipoprotein-cholesterol); TG (triglycerides); CRP (C-reactive protein); Al (atherogenic index) and CRI (coronary risk index).

**DISCUSSION**

In view of the folkloric medicinal use of ingredients of POL4 and the combined formulation in cardiometabolic disorders like hyperlipidemia and hypertension(Duke et al.,...
Fig. 2: Effect of different treatments on endothelium-dependent relaxation in isolated aortae of HFD-fed rats. Data show the means ± SEM of 6 to 7 sets of experiments. ***p<0.001 shows a comparison of HFD with ND (Student’s t-test), while ns (non-significant), ⑦p<0.05 and ⑧p<0.01 show a comparison of all treatments with HFD (One way-ANOVA followed by Tukey’s post-test). POL4 (polyherbal formulation); Ns (N. sativa); Ci (C. intybus); Tfg (T. foenum graecum); Gs (G. sylvestre); ND (normal diet); HFD (high fat diet); Atorva (atorvastatin).

al., 2002), POL4 and its individual components [N. sativa, C. intybus, T. foenum graecum,G. sylvestre] mixed in diet were administered to HFD-fed rats. These interventions prevented raised serum total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), triglycerides (TG), C-reactive proteins (CRP), atherogenic index (AI), coronary risk index (CRI) and fasting blood glucose. Similarly, atorvastatin (suspended in distilled water and administered through oral gavage) also reduced the raised lipid profile. The treatment with POL4 and its ingredients also significantly increased high-density lipoprotein-cholesterol (HDL-C) compared to only HFD-fed rats. The body weight was compromised accompanied by a reduction in daily feed intake in intervention groups compared to HFD fed rats. These findings have clearly suggested that POL4 affects cholesterol/lipoprotein distribution and plays a part in uptake of HDL-C, thus resulting in increased oxidative stress leading to development of obesity, vascular dysfunction and hypertension in experimental animals (Tober, 2003; Olusi, 2002). Increased body weight and/or adipocyte hypertrophy, hepatic steatosis and visceral adiposity are the outcomes of chronic use (Jin et al., 2013). Prolonged use of HFD enhances serum TC, very low density lipoprotein-cholesterol (VLDL-C), LDL-C, AI and CRI (Mopuri and Meriga, 2014). The cholic acid is well known to help cholesterol and fat assimilation and also blocks the conversion of cholesterol into bile acid, hence causing increase in the pool of cholesterol in serum(Ando et al., 2005). Thus, the test material(s) offering protection against hyperlipidemia in HFD-fed animal model may be because of its inhibitory influence on cholesterol and fat absorption from dietary source as observed on the part of POL4 and its ingredients.

Oxidative stress, one of the characteristic features of HFD administration, is a major culprit in development of hypercholesterolemia, endothelium-dependent dysfunction and atherosclerosis. Triggered oxidative stress along with hypercholesterolemia has synergistic role to accelerate the development of vascular resistance, hypertension and associated complications like coronary artery disease, myocardial ischemia and myocardial infarction in animals and humans (Tober, 2003; Lind, 2002). When assessed for effect on systolic blood pressure (SBP) and endothelial function, the parent formulation and its components have decreased SBP and improved the endothelium-dependent relaxation by
causing an increase in ACh-induced relaxation in following orders: Ci>Tfg>POL4Ns. This study reports first time the blood pressure lowering and endothelial modulating properties of POL4 and *C. intybus* in HFD-fed rats, however, *G. sylvestre* has been found relatively the least effective as antihypertensive which is also in line with previous studies for its antihypertensive effect in different animal models (Preuss et al., 1998; Bhansali et al., 2013). Similarly, the weak endothelial modulatory activity has been observed on the part of *G. sylvestre* indicating that its exclusion from current form of the formulation may be suggested if used for the treatment of hypertension.

Numerous studies have reported that high fat diet significantly increases body weight by causing accumulation of adipose tissue (Hariri and Thibault, 2010; Reuter, 2007). The present study has demonstrated that HFD intake induces obesity which was evident by the significant weight gains in HFD-fed animals. The administration of POL4 and its components to HFD-fed rats have exhibited beneficial effects on weight gain and food intake as compared to HFD-fed rats without any treatment. This contribution might be because of the existence of some of the secondary metabolites in *N. sativa* like thymoquinone and tocopherols and in *G. sylvestre* like gymnemic acid and deacetylgymnemic acid which are known for their antiobesity activities (Hasani-Ranjbar et al., 2013; Pothuraju et al., 2014; Manish et al., 2011).

**CONCLUSION**

This study has shown that POL4 and its ingredients possess antihyperlipidemic, endothelium-modulating and antihypertensive properties. The antihyperlipidemic property may be attributed to the inhibitory influence of POL4 on absorption and synthesis of lipids. Thus, this study provides an evidence to the empirical medicinal use of POL4 and its constituents in cardiovascular disorders like hyperlipidemia and hypertension.

**ACKNOWLEDGEMENTS**

The study was carried out during elective rotation of Mr. Abdul Malik, research volunteer, working under supervision of Dr. Malik Hassan Mehmoood and Prof. Dr. Anwarul Hassan Gilani at Department of Biological and Biomedical Sciences (DBBS). We would like to thank Animal Facility staff members for their help in animal experiments. Our sincere gratitude to Department of BBS for funding under the cover of Faculty Research start up fund awarded to Dr. Malik Hassan Mehmoood. We are also thankful to Faculty of Pharmacy, University of Sargodha, Sargodha for providing necessary chemicals to carry out this study. We would like to thank Dr Nauman Aziz for his continuous guidance and helping us to provide test material.

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