HYPOGLYCEMIC EFFECTS OF WHOLE AND FRACTIONATED Azadirachta indica (NEEM) SEED OILS ON ALLOXAN-INDUCED DIABETES IN NEW ZEALAND WHITE RABBITS

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ABSTRACT
Diabetes is a common condition that can contribute to illness, disability and early death worldwide. In an attempt to find remedy to this disease, the hypoglycemic effects of whole and fractionated neem seed oil on alloxan-induced diabetic rabbits were investigated. Oil from the neem seed was soxlet-extracted and some physicochemical properties determined. The whole neem seed oil had an acid value of 4.56±0.11, saponification value of 191.25±0.84, a pH of 4.85±0.15, a refractive index of 1.4653 at 29°C, a density of 0.9048 at 29°C. On fractionation of the whole neem seed oil, the acidic, basic and neutral portion obtained had pH values of 4.35±0.10, 8.50±0.24, 7.75±0.01, respectively. IRspectrophotometric analysis suggests the presence of –OH, -C=O, -CH₃. Rf values obtained from TLC analysis indicates a close relation in chemical components between the whole oil and acidic fraction. On administration of these oils to alloxan-induced diabetic rabbits (2.5 ml/kg p.o.), the whole oil and the acidic portion of oil had very significant (P<0.001) hypoglycaemic effect, the basic portion had a significant effect (P<0.01) whereas the neutral portion had no significant effect (P>0.05) after five days of treatment. The data suggests that the whole neem seed oil and the acidic portion of the neem seed oil could be of benefit in controlling the blood sugar in subjects presenting with diabetes mellitus.

INTRODUCTION
Azadirachta indica, (Family Meliaceae) known as Neem tree is a large evergreen tree, which grows freely all over the coastal, part of the middle belts and the northern parts of Ghana. A variety of biological activities of Azadirachta indica have been reported in scientific literature (van der Nat et al., 1991) and these include the effects on insects; deterrent, feeding inhibition and growth disruption (Warthen, 1979; Schmutterer, 1981; Jacobson, 1986), anti-inflammatory/antirheumatic and anti-arthritic (Bhargava et al., 1970; Okpanyi et al., 1981a; Okpanyi et al., 1981b), antimalaria (Tella, 1977; Rochanakij et al., 1985; Bray et al., 1990), antimicrobial activity (Rao et al., 1986), effect on central nervous system (Singh et al., 1987), antiviral (Andrie et al., 1986), antineoplastic (Pat. Appl. 1983) and antidiabetic effects (Khosla et al., 2000).
Diabetes mellitus (DM) is a multifactorial disease which is characterized by hyperglycemia (Scoppola et al., 2001), lipoprotein abnormalities (Scoppola et al., 2001), raised basal metabolic rate (Avesani et al., 2001; Nawata et al., 2004; Owu et al., 2006), defect in reactive oxygen species scavenging enzymes (Kesavulu et al., 2000) and altered intermediary metabolism of major food substances (Avesani et al., 2001; Unwin et al., 2001; Nawata et al., 2004).

The number of people with diabetes mellitus (DM) worldwide is increasing rapidly. Presently, there are more than 150 million people with diagnosed disease and another 314 million with impaired glucose tolerance, a prediabetic state (International Diabetes Federation, 2010). Close to four million deaths in the 20-79 age group may be attributable to diabetes in 2010 (International Diabetes Federation, 2010) accounting for 6.8% of global all-cause mortality in this age group. It is estimated that the costs of diabetes complications account for between 5% and 10% of total healthcare spending in the world (International Diabetes Federation, 2010). Prevalence estimates of diabetes mellitus (DM) in Africa and Ghana are 3.2% and 3.6% respectively (International Diabetes Federation, 2010).

Morbidity and mortality from the long term complications of DM are high especially among patients with poorly controlled hyperglycaemia. Long term effects of poorly controlled DM include peripheral neuropathy, retinopathy, nephropathy and cardiovascular complications (Hameed et al., 2002).

Oral hypoglycaemic agents are available for managing Type II DM, and insulin for treating mainly Type I DM. Most patients on oral hypoglycaemic agents eventually end up with insulin therapy. Insulin tolerance is a major problem diabetic patients ultimately encounter (Piedrola et al., 2001). This therefore compromises attempts to effectively control hyperglycaemia in patients, thus increasing the risk of patients developing the complications attributable to DM. There is therefore an urgent need for novel compounds with possibly different mechanisms of action to control the condition. As part of the global search for new chemical entities for treating DM, the “fractionated portions” and the “whole neem seed oil” was studied for its hypoglycaemic activity in alloxan-induced diabetic rabbits.

MATERIALS AND METHODS

Plant Sample Collection

Ripe mature fruits of *Azadirachta indica*, were collected from the KNUST Campus between July and August, 2006 and identified by the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana.

Animals

Healthy locally bred adult rabbits of both sexes weighing between 1.8-2.5 kg were used for the experiment. The animals were kept in the Department of Pharmacology animal house under ambient temperature of 25-30°C and relative humidity of 65-87% with a normal 12-hour dark and light cycle and had free access to greens and water *ad libitum*. All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health Services, 1996). The protocols for the study were approved by the Departmental Ethics Committee.

Extraction of Whole Neem Seed Oil

The ripe mature neem fruits collected were sundried for about three weeks. The dried fruits were then crushed to remove the seeds, which were also sundried for about two weeks, then dried in an oven at a temperature range of 55-60°C for an hour. The seeds were then milled into a fine powder using a BlendFast High Power Blender (Model HPB305, Connecticut, USA). About 2.6 kg of the fine powder was refluxed continuously at a temperature of 60°C with 5.2 L of petroleum ether (40-60°C) in a Soxhlet apparatus for 48 hours. The petroleum ether was evaporated off at 60°C after refluxing using Rotavapour (BUCHI) to obtain the neem seed oil which was stored in an amber bottle and kept in the refrigerator.
Fractionation of Whole Neem Seed Oil

Basic Neem Seed Oil

A 500 ml quantity of the neem seed oil extracted was dissolved in 100 ml of petroleum ether (40-60°C). To obtain the basic fraction, the whole oil was extracted with several 10 ml portions of 1M HCl. The combined aqueous acidic extracts were washed once with petroleum ether (40-60°C) to remove traces of whole neem oil that may have been carried over into the aqueous extract. The basic fraction was recovered by cooling the aqueous extracts in an ice bath, basifying it carefully by adding 5M NaOH drop wise with stirring. The precipitated basic fraction was extracted with petroleum ether (40-60°C), dried with anhydrous sodium sulphate, decanted, and the petroleum ether (40-60°C) evaporated. The volume and pH was determined.

Acidic Neem Seed Oil

The neem seed oil from which the basic fraction has been removed was extracted with several portions of 10 ml 1M NaOH. The combined aqueous basic extracts layer was back-extracted with petroleum ether. The acidic fraction was recovered by cooling the alkali extract and acidifying by carefully adding 5M HCl drop wise with stirring. The precipitated oil containing the acidic fraction was extracted with petroleum ether (40-60°C), dried with anhydrous sodium sulphate, decanted, and the petroleum ether (40-60°C) evaporated. The volume and pH were determined.

Neutral Neem Seed Oil

The neem seed oil left after extracting the basic and acidic fractions now contains the neutral components. The neutral fraction was recovered by first washing the oil with several portions of 1M HCl to remove traces of 1M NaOH, and then with distilled water. The resulting solution was dried with anhydrous sodium sulphate, decanted, and the petroleum ether (40-60°C) evaporated off. The volume and pH were determined.

Determination of Physico-chemical Properties of the oils

Organoleptic Tests

A 1 ml quantity of each of the oils was poured onto different evaporating dishes. The smell, colour and taste were noted.

Acid Value

About 10g of the neem seed oil was accurately weighed in a 250 ml conical flask. The neem seed oil was then mixed with 25 ml ether, 25 ml of ethanol (96%), and 1 ml of phenolphthalein indicator, previously neutralised by titrating with few drops of standardised 0.1M KOH, and the flask shaken to dissolve fatty acids which are present in the oil. The resulting mixture was then titrated with standardised 0.1M KOH until the pink colour of the indicator persisted for 15 seconds. The above procedure was repeated twice and the average acid value is then calculated from the titration data.

Saponification Value

Two grams of the neem seed oil was accurately weighed in a 250 ml conical flask. A 25 ml of 0.5 M ethanolic KOH solution was pipetted and added to the conical flask. The conical flask was fitted with a reflux condenser, immersed in a water bath and heated for 1 hour. One millilitre of phenolphthalein indicator was added and the solution was cooled under a tap. The excess KOH in the cold solution was then titrated with standardised 0.5M HCl.

A blank determination was also performed. The above procedure was repeated twice and the average saponification value is then calculated from the titration data.

Density, Refractive Index and pH Determination

The density, refractive index and pH of the whole and fractionated portions of the neem oils were determination at 29°C using a density bottle, a calibrated Abbe’s refractometer (Higler & Watts®, England), and a standardised Delta 350 pH meter (Mettler-Toledo Giegen, Germany) respectively. Three determinations were made for each parameter.
Identification of Components and Functional Groups

A 0.05 ml quantity of each of the oils was run as a film sandwiched between two NaCl discs using capillary tubes and run in a calibrated-FTIR-8201 IR spectrophotometer (Shimadzu, Japan) to identify possible organic functional groups present. Thin layer chromatography was also performed using silica gel H as stationary phase and hexane: isopropanol mixture (9:1). The developed plates were then sprayed with conc. sulphuric acid and chloroform mixture (1:1). The plates were viewed with a C-70G Chromato-Vue Cabinet (Ultra-Violet Products Ltd, UK) at 365 nm.

Induction of Diabetes

The normal plasma glucose concentration of fifty (50) selected healthy rabbits were determined and recorded. The animals were given 2g/kg glucose (Ernest Chemist Ltd, Ghana) orally by gavage just to minimise an almost sudden onset of hypoglycaemia accompanying the injection of alloxan monohydrate. The animals were kept for 30 minutes to ensure absorption of the glucose given and then injected with alloxan monohydrate (BDH, Poole England) in normal saline solution freshly prepared intraperitoneally at a dose of 150 mg/kg (El-Shabrawy et al., 1996).

After 14 days, animals with plasma glucose concentration above 180 mg/dl (10 mmol/L) which were considered diabetic were selected and put into seven groups of five. Each group was treated with either insulin (8.5 units/kg/day), glibenclamide 0.09 mg/kg, whole neem seed oil 2.5 ml/kg, acidic fraction of neem seed oil 2.5 ml/kg, basic fraction of the neem seed oil 2.5 ml/kg, and neutral fraction of the neem seed oil 2.5 ml/kg daily for 5 days. One group was left untreated (control). Dosing of the neem oil was based on preliminary investigation. Plasma glucose concentration was again determined and recorded at one hour and three hours post treatment.

Measurement of Plasma Glucose

A drop of blood obtained from the marginal ear vein of the rabbits (well cleaned with with 70% alcohol and treated with lignocaine), was dropped directly onto the ONE TOUCH™ Test Strips inserted in the ONE TOUCH™ BASIC™ Plus glucose measuring kit. Plasma glucose concentration was read off the meter.

Statistical Analysis

GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Data are presented as mean ± S.E.M. (n=5) and analysed by One-way analysis of variance (ANOVA) followed by Bonferroni’s or Dunnet’s multiple comparisons post hoc test. P ≤ 0.05 was considered statistically significant in all analysis. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

The results of the physiochemical properties of the oils are summarised in Table 1.

On running the IR spectra, the whole neem seed oil had peaks between 3400-3500 cm⁻¹ (medium and broad), 2850-2950 cm⁻¹ (medium and sharp), 1350-1450 cm⁻¹ (medium, sharp and weak), 1150-1200 cm⁻¹ (weak and sharp), and at 3000 cm⁻¹ (sharp), 1750 cm⁻¹ (medium and sharp), and 1725 cm⁻¹ (medium and sharp) (Figure 1). The acidic fraction had peak between 3400-3500 cm⁻¹ (medium and broad), 2850-2950 cm⁻¹, 1350-1450 cm⁻¹ (medium, sharp and weak), 1150-1200 cm⁻¹ (weak and sharp), and at 3000 cm⁻¹ (sharp), 1750 cm⁻¹ (medium and sharp), and 1725 cm⁻¹ (medium and sharp) (Figure 2). The basic fraction of the neem seed oil gave peak at 3000 cm⁻¹ (medium and broad), 2850-2950 cm⁻¹, 1350-1450 cm⁻¹ (strong and sharp), 1350-1450 cm⁻¹ (medium and sharp), 1150-1200 cm⁻¹ and at 1750 cm⁻¹ (Figure 2). The basic fraction of the neem seed oil gave characteristic peaks at 3000 cm⁻¹, 2850-2950 cm⁻¹ and 1450-1500 cm⁻¹ (strong and sharp) and at 1750 cm⁻¹ (medium and sharp), and 1720 cm⁻¹ (medium and sharp) (Figure 3). The neutral fraction gave characteristic peaks at 3000 cm⁻¹, 2850-2950 cm⁻¹ and 1450-1500 cm⁻¹ (strong and sharp) (Figure 4). These peaks obtained indicates that -OH, -C=O, -CH₂, -CH₃ functional groups are present.

After developments of the TLC plates, five spots were observed for the whole neem seed oil.
Table 1: Physicochemical properties of whole and fractionated portions of neem seed oils

<table>
<thead>
<tr>
<th>Neem seed oil</th>
<th>Acid value</th>
<th>Saponification value</th>
<th>pH</th>
<th>Refractive Index</th>
<th>Density</th>
<th>Odour</th>
<th>Taste</th>
<th>Colour and Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>4.56 ± 0.11</td>
<td>191.25 ± 0.84</td>
<td>4.85 ± 0.15</td>
<td>1.4653</td>
<td>0.9048</td>
<td>characteristic neem odour</td>
<td>Bitter</td>
<td>Dark yellow with no precipitates</td>
</tr>
<tr>
<td>Basic</td>
<td>8.50 ± 0.24</td>
<td>1.4659</td>
<td>0.9042</td>
<td></td>
<td></td>
<td>characteristic neem odour</td>
<td>Bitter</td>
<td>Yellowish with small amounts of yellowish gel precipitates</td>
</tr>
<tr>
<td>Acidic</td>
<td>4.36 ± 0.10</td>
<td>1.4651</td>
<td>0.9045</td>
<td></td>
<td></td>
<td>characteristic neem odour</td>
<td>Bitter</td>
<td>Dark yellowish with a lot of yellowish gel precipitates</td>
</tr>
<tr>
<td>Neutral</td>
<td>7.75 ± 0.01</td>
<td>1.4662</td>
<td>0.9038</td>
<td></td>
<td></td>
<td>characteristic neem odour</td>
<td>Bitter</td>
<td>Light yellowish with no precipitates</td>
</tr>
</tbody>
</table>

Fig. 1: IR spectrum of whole neem seed oil obtained using the FTIR-824 spectrophotometer

Fig. 2: IR spectrum of acidic portion of neem seed oil obtained using the FTIR-824 spectrophotometer
oil, four spots were observed each for both the acidic and basic portions and only one spot was observed for the neutral portion. The calculated $R_f$ values are shown in Table 2.

**Table 2: $R_f$ values for detected spots of whole and fractionated neem seed oils**

<table>
<thead>
<tr>
<th>Neem seed oil</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>0.194</td>
<td>0.338</td>
<td>0.500</td>
<td>0.593</td>
<td>0.706</td>
</tr>
<tr>
<td>Basic</td>
<td>(-)</td>
<td>0.343</td>
<td>0.497</td>
<td>0.597</td>
<td>0.710</td>
</tr>
<tr>
<td>Acidic</td>
<td>(-)</td>
<td>0.347</td>
<td>0.499</td>
<td>0.589</td>
<td>0.702</td>
</tr>
<tr>
<td>Neutral</td>
<td>(-)</td>
<td>(-)</td>
<td>0.489</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

(-): no detection

**Fig. 3:** IR spectrum of basic portion of neem seed oil obtained using the FTIR-824 spectrophotometer

**Fig. 4:** IR spectrum of neutral portion of neem seed oil obtained using the FTIR-824 spectrophotometer
In alloxan-induced diabetic rabbits, the no treatment group (control), plasma glucose concentration did not reduce over the five day monitoring period. Compared to the control, plasma glucose concentration for insulin-treated animals were always very significantly low (P ≤ 0.001). The acidic, basic, neutral fractions and whole neem seed oil decreased plasma glucose concentration significantly (P ≤ 0.001) one hour and three hours post treatment over the five days. The rate of reduction was gradual from the day 1 and became quite significant at day two and stabilised to the last day for glucose measurement determined one hour after the drug administration (Figures 5 and 6). The AUC provides a significant decrease in plasma glucose over the five days in comparison to the no-treatment groups (Figures 5 and 6). The rate of decrease in plasma glucose for the various fractions and the whole seed oil was steep from day 0 to day one but stabilised to the last day of the experiment when the measurement of plasma glucose concentration was done

Fig. 5: Effects of the whole neem seed oil and its fractionated portions (neutral, acidic and basic), insulin and glibenclamide on plasma glucose concentration in alloxan-induced diabetic rabbits one hour after drug administration. Values are means ± SEM (n=5). ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni’s post hoc test). †††P<0.001; ††P<0.001; †P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Dunnet’s post hoc test).
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Figure 6: Effects of the whole neem seed oil and its fractionated portions (neutral, acidic and basic), insulin and glibenclamide on plasma glucose concentration in alloxan-induced diabetic rabbits three hours after drug administration. Values are means ± SEM (n=5). **P < 0.01; ***P < 0.001; †††P < 0.001; ††P < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni’s post hoc test). †††P<0.001; ††P<0.001; †P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Dunnet’s post hoc test).

three hours after the drug administration (Figures 5 and 6). The AUC plotted as column graphs confirms this effect. Glibenclamide exhibited no significant decrease over the five days when the glucose measurements were done one hour after the drug administration but the rate of reduction was quite steep three hours after the drug administration (Figures 5 and 6). Insulin, however, caused a significantly steep reduction of the initial glucose concentration from day 0 to day 1 and stabilised for the rest of the five days when the glucose concentration measurements were made one hour and three hours after the drug administration (Figures 5 and 6).

DISCUSSION
The physichochemical properties of the oils were performed to ensure quality control and assurance. The whole neem seed oil had its colour, nature, taste, smell, acid value, saponification value, pH, refractive index, density, con-
forming to the characteristics of the whole neem seed oil that has been reported in earlier works (RYM Exports, 2010)

Diabetes mellitus is characterized by hyperglycemia, which usually produces many complications, such as hyperlipidemia, hyperinsuline mia, hypertension, obesity, atherosclerosis, and even cardiovascular disease (Defronzo et al., 1992; Alberti et al., 1997). Alloxan is a diabeticogenic agent known to induce diabetes mellitus by destroying pancreatic β-cell of the islet of Langerhans where insulin is produced and secreted (Bell et al., 1980). There is therefore a complete lack of insulin (complete β-islet cell destruction) or a reduced secretion of insulin (partial β-islet cell destruction) resulting in the metabolic disorder characterized by hyperglycaemia.

Insulin-treated diabetic animals always have significantly reduced blood glucose because exogenous insulin occupies insulin receptors and cause a reversal of the catabolic features of insulin deficiency and enhances anabolic activity in the liver. It also enhances protein and glycogen synthesis in the muscles and lipogenesis (Herfindal and Gourley, 1996). It is clear from results obtained that glibenclamide was not as effective as insulin in reversing hyperglycemia. Glibenclamide is an insulin secretagogue. Glibenclamide stimulates residual pancreatic β-cells to release insulin resulting in an enhanced peripheral utilization of glucose in the liver, muscles, and adipocytes. Insulin release also reduces secretion of glucagon resulting in the reduction of gluconeogenesis (Katzung, 1992, Boyd, 1998). Glibenclamide, therefore, depends on viable β-cells and insulin receptor sensitivity and activity.

The acidic, basic, neutral fractions and whole neem seed oil decreased plasma glucose concentration significantly. This was comparable to glibenclamide. The neem oils may therefore be acting in a manner similar to the sulphonylureas. It may even be possible that the reduction of plasma glucose caused by the oils may be independent of β-cell activity but on enhanced peripheral utilization of glucose such as the direct stimulation of glycolysis in peripheral tissue, reduction of glycogenolysis in peripheral tissue, the reduction of gluconeogenesis, the slowing of glucose absorption from the gastrointestinal tract, the reduction in plasma glucagon level, and increasing insulin receptor sensitivity and insulin binding to insulin receptors (Katzung, 1992; Clarke and Duncan, 1990; Herfindal and Gourley, 1996).

From the IR spectrophotometry, the oils were found to contain phenolic and carbonyl functional groups which are reducing chemical agents and therefore have antioxidant properties. Phenolic antioxidants are potent free radical terminators and their presence is a good marker of potential antioxidant activity. Polyphenols have the ability to undergo electron donation reactions with oxidizing agents producing stable species (Kang et al., 2005) and thus inhibiting or delaying the oxidation of different biomolecules (Amarowicz, 2005; Seidel et al., 2000). Diabetes has been found to be associated with indices of oxidative damage. Hyperglycemia can lead to the glycation of tissue proteins. Glycation and glucose autooxidation generate hydrogen peroxides, hydroxyl radicals and protein-reactive ketoaldehydes. Hyperglycemia can also lead to increased lipid peroxidation, superoxide production, glycation of the lipoproteins, oxidative DNA damage, and so on. Antioxidants can provide defense against free radical damage. Natural antioxidants may have beneficial implications for diabetes management. Diabetic complications can be prevented or retarded by administration of appropriate antioxidants, in addition to traditional therapeutic principles (Packer et al., 2000).

CONCLUSION

All the fractionated portions and the whole neem seed oil have hypoglycaemic effect. However, insulin has a more significant hypoglycaemic effect than the oils. Therefore these fractions can be used as part of the management regime in the management insulin-dependent diabetes mellitus (IDDM) but can be used non-insulin-dependent diabetes mellitus (NIDDM) which is more prevalent.
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