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Mohamed El Mahad Ibrahim Abdou

Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Olivier Rakotoarison

Laboratoire de Pharmacodynamie, Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Louise Faramalala Rabemandroso

a. Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

b. Laboratoire de Pharmacodynamie, Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Lahadson Stanislas Fulgence

a. Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

b. Laboratoire de Pharmacodynamie, Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Lovasoia Rakotondramasy

Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Anne Wadouachi

Laboratoire de Glycochimie des Antimicrobiens et des Agro-ressources, CNRS UMR 7378 - Institut de Chimie de Picardie CNRS FRE 3085, Université de Picardie Jules Verne, France

Léa Herilala Rasoanaivo

Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Corresponding Author:**Léa Herilala Rasoanaivo**

Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique (LCSN/COB), Faculté des Sciences, Université d'Antananarivo, BP 906 Ankatso, Antananarivo 101, Madagascar

Chemical study of *Phylloxylon xylophyloides* Baker and assessment of its anti-hyperglycaemic activity in Glucose-loaded Normal Rabbits

Mohamed El Mahad Ibrahim Abdou, Olivier Rakotoarison, Louise Faramalala Rabemandroso, Lahadson Stanislas Fulgence, Lovasoia Rakotondramasy, Anne Wadouachi and Léa Herilala Rasoanaivo

Abstract

The present study was carried out to investigate the chemical constituents and the anti-hyperglycaemic activity of *Phylloxylon xylophyloides* Baker. The separations of the chemical compounds of hexane and ethyl acetate extracts on the leaves were carried out using different chromatographic techniques and their structures were elucidated by spectroscopic method including nuclear magnetic resonance. Seven compounds were identified during this investigation. There are trioleine 1, palmitic acid 2, β -sitosterol 3, stigmasterol 4, taraxerol 5, α -amyrine 6, betulinic acid 7. The anti-hyperglycaemic activity of ethanolic extracts of leaves and stem bark of *Phylloxylon xylophyloides* Baker was assessed in glucose-loaded normal rabbits. At a dose of 100, 250 and 500 mg / kg, these ethanolic extracts significantly ($p < 0.05$) lowered the rise in blood glucose with respect to the control group. These results are in accordance with the folk use of this plant.

Keywords: Anti-hyperglycaemic, fabaceae, leaves, *Phylloxylon xylophyloides*, triterpenes, steroids, stem bark

1. Introduction

Diabetes is one of the most prevalent diseases in the world. Approximately 347 million people worldwide had diabetes in 2004. An estimated 3.4 million people died from high levels of fasting blood glucose. More than 80% of diabetes deaths occur in low- and middle-income countries. WHO predicts that by 2030, diabetes will be the seventh leading cause of death worldwide [1]. In order to solve this public health problem in Africa, especially in Madagascar, one of the best solutions is undoubtedly the use and the valorization of the medicinal plants, which are very abundant in the country's forests, and which have already proved their effectiveness in many cases. Many studies are being directed to find anti-diabetic agents from natural sources [2]. The leaves of *Phylloxylon xylophyloides* Baker known as "harahara" are used in traditional medicine to treat diabetes and albuminuria [3]. The phenolic compounds and α -glucosidase inhibitory activity of this plant were reported in a recent study [4]. *Phylloxylon* is a genus endemic to Madagascar of Fabaceae family and it had seven species.

In this work, the isolation and identification of the chemical constituents from hexane and AcOEt extracts of the leaves and the acute antidiabetic activity of the ethanolic extracts of leaves and stem bark of *Phylloxylon xylophyloides* were study.

2. Materials and Methods**2.1 General experimental procedures**

1D (^1H , ^{13}C , DEPT) and 2D (^1H - ^1H COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC) NMR spectra were recorded on a Bruker 600 NMR operating at 600.19 MHz and 125.78 MHz using CDCl_3 as solvent and TMS as an internal standard. Column chromatography (CC) was carried out on silica gel F₂₅₄ (Merck) in glass blades. Thin layer chromatography was performed on precoated TLC plates (Merck, silica 60F254) and visualized under UV light and by spraying with vanillin in H_2SO_4 .

2.2 Plant

Stem bark and leaves of *Phylloxylon xylophyloides* Baker were collected from Joffre Ville in 2007, in the Region of "Diana" in the Northern part of Madagascar. This species was identified at the Department of Botany of the Botanical and Zoological Park of Tsimbazaza (Antananarivo).

A voucher specimen was deposited in the "Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique" at the Faculty of Sciences, University of Antananarivo for future references.

2.3 Animals

Animals were only used once and in accordance with the ethical guidelines for the care of laboratory animals.

Female Swiss mice weighing 14 to 20 g were used for toxicity studies. They were provided by IMVAVET (Institut Malgache des Vaccins Vétérinaires) and were acclimatized to the laboratory conditions for a week before use. They were fastened overnight before each experiment and had free access to water.

Healthy adult male and female rabbits weighing between 1600-2100 g were chosen as experimental animals during the antihypoglycaemic test. They were previously subjected to a fast hydration of 12 hours.

2.4 Extraction

Dried stem bark and leaves of *Phylloxylon xylophyloides* Baker were reduced in to a fine powder using a mechanical grinder. The powder of stem bark or leaves (300 g) was extracted by maceration with a mixture of Ethanol-water (80:20) (1500 ml) for 10 days, and concentrated to dryness under vacuum at a low pressure and low temperature of 60°C to give ethanolic extracts of stem bark and leaves.

The powder of leaves (300 g) partitioned successively with hexane, ethyl acetate and methanol by maceration to give respectively hexane, ethyl acetate and methanol extracts.

2.5 Biological activity

2.5.1 Acute toxicity study

The test was performed according to the method of Onifade *et al.* in 2011 [5]. Mice were randomly assigned to a control or treatment groups (3 animals per group). Ethanolic extracts of leaves and bark were tested separately at a dose of 250, 500 and 1000 mg / kg each. Extracts were given orally to test groups, while the control group received distilled water at the same volume (10 ml/kg b.w), then all the mice were kept under observation for 6 hours on the first day, and thereafter observed daily for 3 days.

2.5.2 Anti-hyperglycaemic activity

The test was performed according to the method of Keita, A. *et al.* in 1998 [6]. The overnight fasted rabbits were divided into 8 groups of three animals each. Then all rabbits were treated orally with a single dose by using a stomach tube. Group 1 served as normal control group and received distilled water; group 2 served as standard and was treated with glibenclamide (10 mg/kgb.w); groups 3 to 5 received ethanol extract of leaves of *Phylloxylon xylophyloides* Baker at a dose of 100, 250 and 500 mg / kg respectively. The same doses of bark ethanolic extract were administered to the animals of groups 6 to 8. After 1 h of drug administration, the rabbits were orally treated with glucose (2 g/kg). The blood glucose levels were measured beforehand, then, 30, 60 and 90 minutes after glucose feeding, with an Accu-check glucometer performed with a few drops of blood punctured in the marginal vein of the ear [7]. The blood glucose was calculated on an average of 3 rabbits and the results were expressed in mg / 100 ml of blood \pm e.s.m.

2.5.3 Statistical analysis

Data were assessed by statistical analysis using unpaired

Student's 't' test and the "Excel 2010" software. P values < 0.05 were considered as significant.

2.6 Isolation procedure

The hexane extract (4.28 g) was subjected to column chromatography over silica gel (180 g silica gel 60, 100x4.5 cm). A total of 215 fractions were eluted with mixtures of hexane/ AcOEt (from 100:0 to 0:100). The fractions were monitored using TLC and viewed under UV light (254 and 365nm) and by spraying with 50% H₂SO₄ reagent followed by heating at 100°C. The fractions were combined on the basis of TLC profiles and purified with MeOH. The fraction [38] (14 mg) over silica gel 60 eluted with hexane-ethyl acetate (99:1) showed one spot containing a mixture of β -sitosterol 3 and stigmasterol 4. Fraction [44] (10 mg) from hexane-ethyl acetate (95:5) exhibited one TLC spot containing taraxerol 5. Trioleine 1 (14 mg) was obtained in fraction 60 from hexane-ethyl acetate (90:10).

The ethyl acetate extract (1.2 g) was subjected to column chromatography over silica gel (60g silica gel 60, 57x4 cm). A total of 415 fractions were eluted with mixtures of hexane/ ethyl acetate (from 100:0 to 0:100). The fractions were monitored using TLC and viewed under UV light (254 and 365nm) and by spraying with 50% H₂SO₄ followed by heating at 100°C. The fractions were combined on the basis of TLC profiles and purified with MeOH. The fractions [73-79] (10 mg) eluted with hexane showed one spot containing a mixture of β -sitosterol 2, stigmasterol 3 and palmitic acid 2. Combined fractions [80-84] (10 mg) eluted with hexane-ethyl acetate (99:1) exhibited one TLC spot containing a mixture of α -amyryne 6 and taraxerol 5. The fraction 101 (10 mg) eluted with hexane-ethyl acetate (92.5:7.5) yielded one spot for a taraxerol 5. Betulinic acid 7 (10 mg) was obtained in fractions (102-103).

2.7 Physical and spectroscopic data

2.7.1 Trioleine 1: ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 5.35(4H, H-9,H-10), 5.24(1H, H-2glycerol), 4.32 (2H,H-1 β ,H-3 β glycerol), 4.16(2H, H-1 α ,H-3 α glycerol), 2.02(12H,H-8,H-11), 1.62(6H,H-3), 1.27(6H, H-4), 0.88(9H,H-18); ¹³C NMR (CDCl₃, 125.78 MHz) δ (ppm):173.2(C-1,C-1'), 172.9(C-1'), 129.0(C-9, C-10), 66.7(C-2 glycerol), 62.1(C-1,C-3 glycerol), 34.2(C-2), 31.9(C-8,C-11), 29.9(C-4, C-5, C-6, C-7, C-12, C-13, C-14, C-15), 27.2(C-3) 24.8(C-16), 22.7(C-17), 14.2(C-18).

2.7.2 Palmitic acid 2: ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 0.88 (3H, H-16); ¹³C NMR (CDCl₃, 125.78 MHz) δ (ppm):178.0(C-1), 33.7(C-2), 31.9(C-14),29.7(C-9 to C-12), 29.6(C-6 to C-8), 29.4(C-13), 29.3(C-5), 29.0(C-4), 24.7(C-3), 22.7(C-15), 14.1(C-16).

2.7.3 β -sitosterol 3: ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 5.35(1H, H-6),3.49(1H,H-3), 1.01(3H, s, H-19), 0.92(3H, s, H-21), 0.85(3H, t, H-29), 0.83(3H, d, H-26), 0.79(3H, d, H-27), 0.68(3H, s, H-18) ¹³C NMR (CDCl₃, 125.78 MHz) δ (ppm): 140.7(C-5), 121.7(C-6), 71.7(C-3), 56.8(C-14), 56.0 (C-17), 50.1(C-9), 45.8(C-24), 42.3(C-13), 42.2(C-4), 39.7(C-12), 37.2(C-1), 36.1(C-20), 36.5(C-10), 33.9(C-22), 31.9(C-7),31.8(C-8), 31.6(C-2), 29.1(C-25), 28.2(C-16), 24.3(C-15), 26.0(C-23), 23.0(C-28), 21.0(C-11), 19.8(C-26), 19.4(C-19), 18.9(C-27), 18.7(C-21), 11.8(C-29), 11.7(C-18).

2.7.4 Stigmasterol 4: ¹H NMR (CDCl₃, 600 MHz) δ (ppm):

5.39(1H, H-6), 5.14(1H, H-22), 4.98(1H, H-23), 3.49(1H, H-3), 1.02(3H, d, H-21), 1.01(3H, s, H-19), 0.84(3H, d, H-27), 0.81(6H, m, H-29, H-26), 0.79(3H, s, H-18); ^{13}C NMR (CDCl_3 , 125.78 MHz) δ (ppm): 140.7(C-5), 138.3(C-22), 129.2(C-23), 121.7(C-6), 71.7(C-3), 56.7(C-14), 55.8(C-17), 51.2(C-24), 50.3(C-9), 42.2 (C-4, C-13), 40.5(C-20), 39.6(C-12), 37.2(C-1), 36.5(C-10), 31.9(C-7, C-25), 31.8(C-8), 31.6 (C-2), 28.9(C-16), 25.4(C-28), 24.3(C-15), 21.2(C-27), 21.0(C-11, C-21), 19.4(C-19), 19.0(C-26), 12.2(C-18), 12.0(C-29).

2.7.5 Taraxerol 5: ^1H NMR (CDCl_3 , 600 MHz) δ (ppm): 5.47(1H, t, H-15), 3.12(1H, t, H-3), 1.54(2H, d, H-16), 1.47(2H, m, H-2), 1.02(3H, s, H-27), 0.91 (3H, s, H-23), 0.88(3H, s, H-29), 0.86(3H, s, H-25), 0.84(6H, s, H-26, H-30), 0.75(3H, s, H-28), 0.73(3H, s, H-24); ^{13}C NMR (CDCl_3 , 125.78 MHz) δ (ppm): 158.0(C-14), 116.8(C-15), 79.0(C-3), 55.5(C-5), 49.3(C-9), 48.7(C-18), 41.3(C-7), 38.9(C-8), 38.7(C-4), 38.0(C-10), 37.7(C-1, C-16), 37.5(C-13), 36.7(C-19), 35.8(C-17), 35.1(C-22), 33.7(C-12), 33.4(C-29), 33.1(C-21), 29.9(C-28, C-30), 29.8(C-20), 28.8(C-20), 28.0 (C-23), 27.2(C-2), 25.9(C-26), 21.4(C-27), 18.8(C-6), 17.5(C-11), 15.5(C-24), 15.4(C-25).

2.7.6 α -amyrine 6: ^1H NMR (CDCl_3 , 600 MHz) δ (ppm): 5.06(1H, d, H-12), 3.13(1H, H-3), 1.00(3H, s, H-27), 0.94(3H, s, H-26), 0.93(3H, s, H-23), 0.88(3H, s, H-25), 0.85(3H, d, H-30), 0.73(6H, H-24, H-28), 0.72(3H, s, H-29); ^{13}C NMR (CDCl_3 , 125.78 MHz) δ (ppm): 139.5(C-13), 124.4(C-12), 79.0(C-3), 59.1(C-18), 55.1(C-5), 47.7(C-9), 41.8(C-14), 41.5(C-22), 40.7(C-8), 40.0(C-20), 39.7(C-4), 39.6(C-19), 37.7 (C-1), 36.5 (C-10), 33.7 (C-17), 32.8(C-7), 31.2(C-21), 28.7(C-28), 28.0(C-23), 27.3(C-15), 26.6(C-16), 23.4(C-2), 23.3(C-11), 23.2(C-27), 21.4(C-30), 18.7(C-6), 17.4(C-29), 16.8(C-26), 15.6(C-25), 15.3(C-24).

2.7.7 Betulinic acid 7: ^1H NMR (CDCl_3 , 600 MHz) δ (ppm): 4.69(3H, H-29), 4.57(1H, H-29), 3.48(H, 1H-3), 1.65(3H, H-30), 0.94(3H, H-27), 0.88(3H, H-23), 0.86(3H, H-26), 0.77(3H, H-25), 0.67(3H, H-24), ^{13}C NMR (CDCl_3 , 125.78 MHz) δ (ppm): 177.2(C-28), 150.4(C-20), 109.6(C-29), 76.8(C-3), 55.5 (C-5), 54.7(C-17), 49.9(C-9), 48.8(C-18), 46.5(C-19), 42.0(C-14), 40.0(C-8), 38.4(C-4), 38.2 (C-10), 37.5(C-1), 36.5(C-13), 36.3(C-22), 33.7(C-7), 31.5(C-16), 30.0(C-21), 28.9(C-15), 28.2(C-23), 26.9(C-2), 24.9(C-12),

20.4(C-11), 18.6(C-30), 18.0(C-6), 15.9(C-25), 15.8(C-24), 15.7(C-26), 14.1(C-27).

3. Results

3.1 Extraction

The yields obtained from the maceration with ethanol 80% carried out on powders of the leaves and stem bark of *Phylloxylon xylophylloides* Baker were 13.16% and 4.83% respectively. These extracts were used for biological activity. Maceration of leaves powder (300 g) using the solvent in increased polarity yielded 2.94g (0.98 %) of hexanic extract, 4.10 g (1.41 %) of ethyl acetate extract and 8.82 g (3.15 %) of methanolic extract.

3.2 Acute toxicity

There was no mortality observed in any of the animals, which lived up to 3 days, following the administration of the ethanolic extracts of stem bark and leaves of *Phylloxylon xylophylloides* at a single dose level of 250, 500 and 1000 mg/kg. However, either leaves or bark extracts at 500 and 1000 mg/kg dosage induced a decrease in locomotor activity in treated mice. These signs began to appear 3 minutes after the administration and persisted for 30 minutes. After 30 minutes, all the animals were normal.

3.3 Anti-hyperglycaemic activity

The administration of glucose (2 g / kg) to normal rabbits (control group) led to transient hyperglycemia and the highest blood glucose level (235 ± 13.47 mg / 100 ml) was obtained after 30 minutes. When compared with their corresponding glucose levels at 30 minutes (Figure 1), 100; 250 and 500 mg / kg of ethanolic extract of stem bark significantly ($p < 0.05$) lowered the rise in blood glucose respectively to 172.67 ± 25.11 ; 168.34 ± 3.11 and 151.50 ± 1.50 mg / 100 ml. Furthermore the same doses of ethanolic extracts of leaves caused a marked significant ($p < 0.05$) reduction (Figure 2): 186.34 ± 9.11 ; 178.67 ± 5.11 and 178.33 ± 12.89 mg / 100 ml respectively. The ethanolic extract of stem bark at 500 mg/kg showed the highest anti-hyperglycaemic activity. On the other hand, while comparing between groups, the effect of ethanolic extracts of stem bark and leaves had no significant difference ($P > 0.05$). In addition, the administration of 10 mg/kg of glibenclamide caused a significant ($P < 0.05$) reduction in the rise of blood glucose level at 30 and 60 minutes.

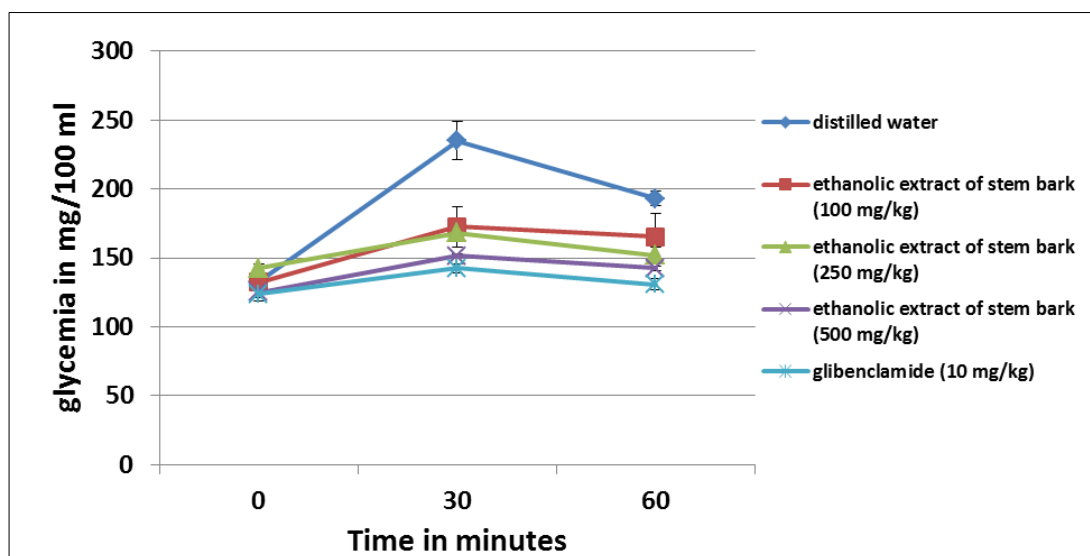


Fig 1: Anti-hyperglycaemic activity of the ethanolic extract of stem bark of *Phylloxylon xylophylloides* Baker (mg/100 ml; n = 3).

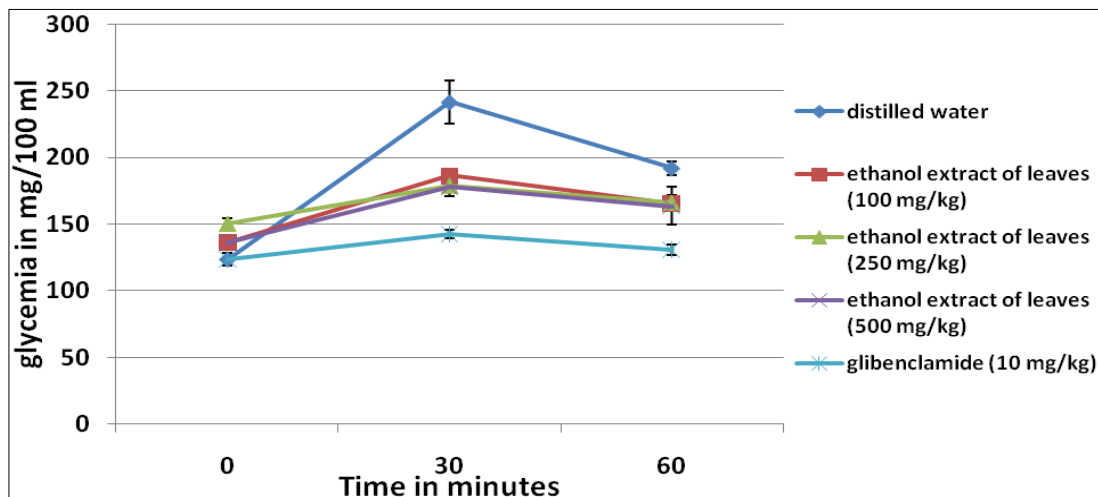


Fig 2: Anti-hyperglycaemic activity of the ethanolic extract of leaves of *Phylloxylon xylophyloides* Baker. (mg/100 ml, n = 3).

3.3 Chemical study

The hexane and ethyl acetate extracts of the leaves of *Phylloxylon xylophyloides* Baker yielded fatty acid, triglyceride, triterpenoids and steroids by silica gel chromatography. Assignments of the ^1H and ^{13}C -NMR of these compounds were accomplished from ^1H - ^1H COSY, ^1H -

^{13}C HSQC and ^1H - ^{13}C HMBC experiments. These compounds were identified by comparison of their spectral data with those of trioleine 1 [8], palmitic acid 2 [9], β -sitosterol 3, stigmasterol 4 [10], taraxerol 5 [11, 12], α -amyrine 6 [13, 14] and betulinic acid 7 [15] (figure 3) reported in the literature.

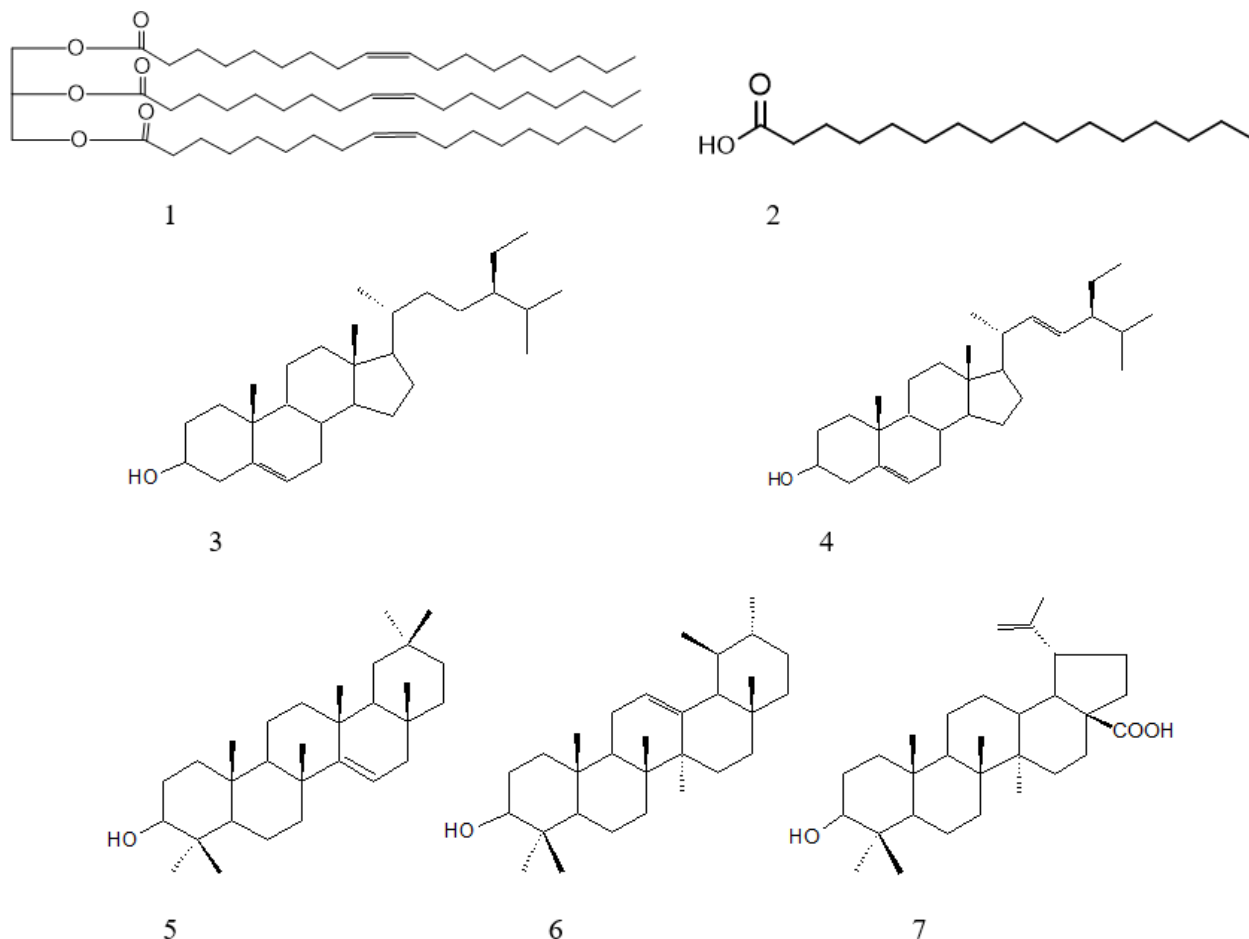


Fig 3: Structure of isolated compounds from the leaves of *Phylloxylon xylophyloides* Baker

4. Discussion

The results of this study revealed that the ethanolic extracts of stem bark and leaves of *Phylloxylon xylophyloides* exhibited a significant anti-hyperglycaemic activity, and this justifies its popular use in traditional management of diabetes. These ethanolic extracts exhibited a low toxicity, according to the

Hodge and Sterner toxicity scale [16]. The compounds isolated from hexane and ethyl acetate extracts of the leaves of this species, found in many species, have never been isolated before from *Phylloxylon* species. Although bioassays were not conducted on these isolated compounds, there were previous studies reporting on their biological activities. It is reported

that triterpenoids constitute the active biological principles of most medicinal plants with anti-hyperglycaemic and anti-diabetic properties [17], 23. Taraxerol is also known to be of benefit in treating diabetes [18, 19]. β -Sitosterol shows an insulin releasing effect and a prevention of oxidative damage through its antioxidant activity [20]. Betulinic acid treatments improve insulin sensitivity as well as pancreas histopathology and reduce blood glucose and α -amylase [21]. The observed potential anti-hyperglycaemic activity may be partly due to the large presence of triterpenoids and steroids in this plant species; not surprising therefore to observe activities in ethanolic extract since it contained triterpenoids taraxerol as a major constituent [22].

5. Conclusion

The results of the present study showed that *Phylloxyton xylophyloides* Baker has anti-diabetic properties and this justifies the traditional use of this plant in the treatment of diabetes.

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