BIOASSAY GUIDED ISOLATION OF ACTIVE PHYTOCHEMICALS FROM *HYPHAENETHEBAICA* (L) MART FRUIT PULP METHANOL EXTRACT RESPONSIBLE FOR HYPOGLYCAEMIC ACTIVITY

[1]Shehu BB, [1]Zanna H, [2] Tukur MA [3]Hamidu U, [1]Gidado A.

[1]Department of Biochemistry, University of Maiduguri,
[2] Department of Human Physiology University of Maiduguri,
[3]Department of Chemistry, University of Maiduguri.

Correspondence and reprint request to: **Dr Binta B Shehu,** Department of Biochemistry, Faculty of Sciences University of Maiduguri, PMB 1069, Maiduguri, Nigeria

ABSTRACT

Background: Active phytochemical responsible for hypoglycaemicactivily from *Hyphaenethebaica* (L) Mart were separated using Bioassay guided fractionation. **Methodology:** Five hundred grams (500g) methanolic fruit extract of *H. thebaica*was suspended in water, filtered and sequentially partitioned with hexane, chloroform, ethyl acetate and n-butanol. The n-butanol fraction was subjected to column chromatography, sub-fraction A,B,C,D,E,F and G were obtained. Further separation using preparative TLC of fraction C afforded sub-fraction C_1, C_2, and C_3. Finally C_1 gaveRF value. Diabetes was induced to albino rats of both sexes by intra muscular injection of 120mg/kg Alloxan monohydrate. The diabetic rats were grouped according to the number of fractions and sub-fractions with 3 rats each. Each fraction was tested for hypoglycaemic activity. The one with highest activity was used for next study and phytochemical constituents analysed. **Results**: The phytochemical screening of70% methanol extract of *Hyphaenethebaica*fruit pulp were reported to contain saponins, tannins, terpenoids, cardiac glycosides with the exception of alkaloid, combined anthraquinone, free anthraquinone and soluble starch. The n-butanol portion showed hypoglycaemic activity (66.37±1.03% reduction) compared to other fractions at 400mg/kg. The n-butanol portion similarly contained phytochemicals found in crude extract except forsaponins which is absent. The column fraction C of the n-butanol portion has maximum reduction (45.33± 2.80%) of fasting blood glucose of diabetic rats at a lower dose of 200 mg/kg. Sub-fraction C_1 has more hypoglycaemic activity of 60.50%while phytochemical evaluation showed the presence of flavonoids. **Conclusion:** Flavonoids may be responsible for the observed hypoglyceamic effect of *H. thebaics* fruit pulp.

Keywords: Phytochemicals, *Hyphaenethebaica,* Bioassay guided isolation, Hypoglyceamic activity.

INTRODUCTION

Hyphaenethebaica (L) Mart is a plant used for its fruits, leaves and roots for medicinal purpose in the North East Arid zone of Nigeria. The plant, *Hyphaenethebaica* is a member of the *Palmae* (*Arecaceae*) family.[1] Its local names include Egyptian doum palm, goruba, kongor or bur. The fruit is being used as condiment to enhance flavor and tastes like ginger, hence the name in English, 'ginger bread' in some places.

The fruits are traditionally used in the management of hypertension and as haematinic agent.[2] It has been reported that high concentration of ethanol extracts of the plant is hypolipidemic, hepatotoxic and nephrotoxic.[3] However using aqueous pulp extracts of *Hyphaene thebaica*(L) Mart, the extactwas found to be hypolipidemic but non toxic to both liver and kidney.[4] The chloroform extract of the fruits improved spermatic count of male rats at low concentration but could decrease the count at high concentration.[5] The aqueous fruit extract of *Hyphaenethebaica* has significant antifungal properties on *Candidaalbicans*, *Microsporumgypseum*, *Trichlorophytonrubrum*, *Mucor* sp., *Fusariumsolani* and *Aspergillusniger.*[6] Ina study using aqueous suspension of the root of *Hyphaenethebaica (L)* Mart,it was reported that the extract was hypocholesterolemic, hepato and nephrotoxic.[7] Methanolextract of the fruit pulp of *H.*

thebaica(L)Mart reduced fasting hyperglycaemia[8] and increased levels of ALP and ALT at higher dose in albino rats.[9]

Knowledge of the phytochemical constituents of the plant is desirable not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials like tannins, oils, gums, and as precursor used for the synthesis of complex chemical substances. In addition, the knowledge of phytochemical constituents of plants would further be valuable in discovering and authenticating folkloric remedies.[10] In this study,methanol fruit pulp extract of *H. thebaica*was subjected to bioassay guided fractionation to ascertain the phytochemical compound responsible for hypoglycaemic activity

MATERIALS AND METHOD

Sample Collection: Freshripe fruit of *Hyphaenethebaica,* was collected from Konduga Local Government Area of Borno state, Nigeria. The plant was identified by plant taxonomist from the Department of Biological Science, University of Maiduguri. The fruit were cleaned, debris removed, fleshy mesocarp were separated from the hard endocarp, shade dried and ground into powder using mortar and pestle.

Methanol Extract Preparation: *Hyphaenethebaica* fruit pulp powder (500g) was macerated with 2.5 liters of 70% methanol in a glass jar for 2 days at room temperature (37°C), the extract was filtered with filter paper and concentrated to dryness under reduced temperature and pressure on a rotary evaporator to obtain gummy extract.

Experimental Animals: White Wistar strain albino rats of both sexes weighing between 120 and 200g were used for the study. The rats were obtained from the animal house of the Department of Veterinary Phamacology University of Maiduguri and maintained under standard conditions of light and temperature with the minimum 12hours dark and light cycle. The animals were fed standard rats diet (growers mesh, ECWA feed Nigeria Ltd) and water *ad-libitum.*

Induction of Diabetes: Diabetes was induced by a single intramuscular injection of 120mg/kg alloxan monohydrate dissolved in cold normal saline after an overnight fast.[11] After two weeks, surviving rats with blood glucose of more than 200 mg/dl were considered diabetic and used for the study.

Determination of Blood Glucose: Blood glucose concentration (mg/dl) was estimated by the glucose oxidase enzymatic method using commercial glucometer and test strips (Accu-chek Advantage II Glucose, Roche U.S.). It was determined based on the principle of Rhenry and Kirk.[12]

Phytochemical Screening: Methanol fruit extract, fractions obtained from solvent, with highest hypoglyceamic activity from the silica gel column activity were subjected to phytochemical screening which included test for carbohydrates, flavonoids, tannins, saponin, terpenes, glycosides, phlobatannins and steroids, using standard methods described by Trease and Evans.[13]

Bioassay Guided Isolation of Active Compound: Five hundred grams (500g) of methanol fruit extract of *H. thebaica* was suspended in water, filtered and sequentially partitioned with hexane, chloroform, ethyl acetate and n-butanol. The various fractions were tested for hypoglycaemic activity and the fraction that had highest activity was found to be n-butanol fraction and later subjected to column chromatography packed with silica gel mesh size (60-120G). The n-butanol fraction was eluted gradiently from the column using 1 litre each ethylacetate :methanol mixture in ratio1:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:1. The fractions were obtained coded A,B,C,D,E,F, and G. Fraction C eluted with 7:3ethylacetate:methanol exhibited the highest hypoglycaemic activity. The fraction C was subjected to preparative TLC. The ethyl acetate and methanol ratio (8:2) was used as a solvent system to run the preparative TLC. The chromatogram gave three fractions (C_1, C_2 and C_3) which were scraped, dissolved in methanol and filtered. Each fraction was tested for hypoglycaemic activity and phytochemical analysis conducted[14].

Statistical analysis

The data obtained were presented as Mean and Standard error of mean (Mean ± SEM). Differences among mean were analysed using analysis of variance (ANOVA), by computer statistical software graphpadinstat® (2003). Probability value (P value) ≤ 0.05 was considered significant

RESULT

The phytochemical evaluation of methanol*H. thebaica*fruit pulp extract showed the presence of flavonoids, saponins, tannins, terpenoids, cardiac glycosides and carbohydrates and the absence of alkaloids, anthraquinones and soluble starch outlined in Table 1. n-butanol soluble portion had the highest hypoglacemic activity compared to other portions. This portion reduces the fasting blood glucose of diabetic rat from 451.33± 15.21 mg/dl to 239.00± 15.25 mg/dl representing 66.37± 1.03% reduction at 400mg/kg dose (Table 2). The phytochemical screening of the n-butanol showed the presence of flavonoids, tannins, terpenoids carbohydrates and absence of saponins (Table 3) .

The n-Butanol fraction was subjected to column chromatography, seven sub-fractions were obtained. The sub-fraction eluted with ethyl acetate-methanol (7:3) contains the most hypoglycaemic activity (fraction C)with 45.33 ± 2.80% maximum reduction of fasting blood glucose of diabetic rats at 200mg/kg which was significantly ($P<0.05$) higher compared with fractions A,B ,D,E,F andG (Table 4).

The phytochemical screening of the sub-fraction showed both the presence of carbohydrates , terpenoids and flavonoids and absence of tannins(Table 5). On further fractionation of fraction C using preparative TLC, three components were obtained (C_1, C_2 and C_3 which had Rf values of 0.78, 0.67, and 0.54 respectively. The fraction C_1 had more activity producing60.5% reduction at 100mg/kg (Table 6) with phytochemical analysis showing the presence of flavonoids.

Table 1: Phytochemical Constituent of Methanolic Extract of *Hyphaenethebaica*(L) Mart Fruit Pulp

	Tests	Result
1	Carbohydrates	
	a) Reducing sugar test	+
	b) Fehling's test	+
	c) Barford's test	–
	d) Molish test	+
	e) Ketoses	+
	f) pentoses	–
2	Pholabutannins	–
3	Cardiac glycosides	
	a) Lieberman – Burchard test	+
	b) Salkowskitest	+
4	Soluble starch	–
5	Free Anthraquinones	–
6	Combined Anthraquinones	–
7	Tannins	
	a) Ferric chloride	+
	b) Lead acetate	+
8	Flavonoids	
	a) Ferric chloride	+
	b) Sodium hydroxide	+
	c) Lead acetate	+
	d) Shinoda's test	+
9	Alkaloids	
	a) Dragndorff's	–
	b) Meyers	–
10	Terpenoids	+
11	Saponins	
	a) Frothing	+
	b) Fehling	+
12	Cardenolides	
	a) Legal test	–
	b) Keller – killiani test	+

Key: + Present, – Absent

Table 2: Effect of Partitioned Fraction from *H. thebaica* (L.) Mart Fruit Pulp Methanolic Extract (400mg/kg) on Fasting Blood Glucose (mg/dl) on Alloxan Induced Diabetic Rats (n=3)

	Period of treatment (hrs)					
Fraction	0	1	2	4	6	% reduction
Ethylacetate	308.67±12.99	292.57±15.60	280.57±15.60	262.67±16.92	249.62±14.10	19.13±1.21
n-Butanol	451.33±15.21	351.21±16.24	344.00±14.51	328.67±18.23	239.00±15.25	66.37±1.03*
Residual aqueous	282.33±18.04	279.67±17.51	276.67±16.13	270.33±17.49	263.67±18.53	6.61±1.02

* Significantly higher (P<0.05) compared with percentage maximum reduction of other fractions

Table 3: Phytochemical Constituent of n-Butanol Fraction from Methanolic Extract of the *Hyphaenethebaica* (L.) Mart Fruit Pulp

	Tests	Result
1	Carbohydrates	
	a) Reducing sugars test	+
	b) Fehling's test	–
	c) Barford'stest	–
	d) Molish test	+
	e) Ketoses	+
	f) Pentoses	–
2	Pholabutannins	–
3	Cardiac glycosides	–
	a) Lieberman Burchard	–
	b) Salkowski	+
4	Soluble starch	–
5	Free anthraquinone	–
6	Combined Anthraquinone	–
7	Tannins	
	(a) Ferric chloride	+
	(b) Lead acetate	+
8	Flavonoids	
	(a) Ferric chloride	+
	(b) Sodium hydroxide	+
	(c) Lead acetate	+
	(d) Shinoda	+
9	Alkaloids	
	(a)Dragendorffs	–
	(b)Mayers	–
10	Terpenoids	+
11	Saponins	
	(a)Frothing	–
	(b)Fehling	–
12	Cardenolides	
	a) Legal test	–
	b) Keller – Killiani	+

Key: + Present, – Absent

Table 4: Effects of Fractions from Column Chromatography on Silica-gel of n-Butanol Fraction on Fasting Blood Glucose (mg/kg) level of Alloxan Induced-diabetic Rats n=3

Fractions (200mg/kg)	Period of Treatment (hr)					
	0	1	2	4	6	% reduction
A	220.00±6.23	218.23±6.45	210.00±4.21	219.42±6.02	211.12±2.13	4.04±1.24
B	205.80±13.62	219.60±12.26	203.40±12.04	190.45±10.25	180.00±10.42	12.54±2.02
C	300.00±10.25	272.45±8.62	251.83±8.66	182.56±9.23	164.00±7.81	45.33±2.80*
D	250.40±6.46	238.00±5.63	230.21±10.25	228.00±7.52	220.32±6.07	12.01±0.19
E	310.20±15.20	304.00±13.45	296.20±14.60	291.00±13.72	285.24±12.41	8.05±2.74
F	240.80±12.24	238.00±12.72	220.52±10.43	210.33±5.72	208.11±5.75	13.58±2.33
G	223.00±4.65	220.23±5.02	219.00±4.01	217.60±4.60	217.33±4.42	2.54±0.13

* Significantly higher (P<0.05) compared with the percentage maximum reduction of other fractions.

Table 5: Phytochemical Constituent of Fraction C from Column Chromatography on Silica-gel of n-Butanol Fraction

	Test	Result
1	Carbohydrates	
	a) Reducing sugars	+
	b) Fehlings	–
	c) Barfords	–
	d) Molish test	–
	e) Ketoses	+
	f) Pentoses	–
2	Pholabutannins	–
3	Cardiac glycosides	–
	a) Lieberman Burchard	–
	b) Salkowski	–
4	Soluble starch	–
5	Free anthraquinone	–
6	Combined Anthraquinone	–
7	Tannins	
	(a) Ferric chloride	–
	(b) Lead acetate	–
8	Flavonoids	
	(a) Ferric chloride	–
	(b) Sodium hydroxide	+
	(c) Lead acetate	–
	(d) Shinoda	+
9	Alkaloids	
	(a)Dragendorffs	–
	(b)Mayers	–
10	Terpenoids	+
11	Saponins	
	(a)Frothing	–
	(b)Fehling	–
12	Cardenolides	
	a) Legal test	–
	b) Keller – Killiani	–

Key: + Present, – Absent

Table 6 : Effect of Fractions (C_1, C_2 and C_3) Collected from Scraped Preparative TLC Plates of fraction C on Fasting Blood Glucose (mg/dl) of Alloxan Induced Rats (n=3)

Treatment 100mg/kg	Period of treatment (hrs)					
	0	1	2	3	4	% reduction
C_1	265.42±12.28	260.34±11.16	243.62±14.29	194.62±13.48	104.82±16.00	60.51±1.02*
C_2	268.30±16.30	290.66±18.56	258.21±14.30	242.44±12.19	240.28±14.20	9.23±2.01
C_3	252.21±10.34	262.31±14.02	260.60±16.21	256.45±12.23	250.41±14.23	0.88±0.21

C_1 *($p<0.05$) compared with percentage reduction of other fraction

Table 7: Qualitative Phytochemical Constituent of Fraction C_1Collectedfrom Scraped Preparative TLC Plates of Fraction C

	Secondary metabolites	Results
1	Carbohydrates	
	a) Reducing sugars	+
	b) Fehlings	–
	c) Barfords	–
	d) Molish test	–
	e) Ketoses	+
	f) Pentoses	–
2	Pholabutannins	–
3	Cardiac glycosides	–
	a) Lieberman Burchard	–
	b) Salkowski	–
4	Soluble starch	–
5	Free anthraquinone	–
6	Combined Anthraquinone	–
7	Tannins	
	(a) Ferric chloride	–
	(b) Lead acetate	–
8	Flavonoids	
	(a) Ferric chloride	–
	(b) Sodium hydroxide	+
	(c) Lead acetate	–
	(d) Shinoda	+
9	Alkaloids	
	(a)Dragendorffs	–
	(b)Mayers	–
10	Terpenoids	–
11	Saponins	
	(a)Frothing	–
	(b)Fehling	–
12	Cardenolides	
	a) Legal test	–
	b) Keller – Killiani	–

Key: + Present, - Absent

DISCUSSION

Medicinal plants constitute a rich source of bioactive chemicals that are largely free from adverse effects and have excellent pharmacological action; they could lead to the development of new classes of possibly safer anti diabetic agents. The methanolic fruit pulp extract of *H. thebaica*was subjected to bioassay guided fractionation. The presence of carbohydrates, tannins, saponins, flavonoids, terpenoids, cardiac glycosides, absence of alkaloid and anthraquinones in the methanol fruit pulp extract of *Hyphaenethebaica* agrees with ealier findings.[15] The use of some plants as medicinal plant is due to the presence of flavonoids and saponins.[16] Hence the use of the extract, which is rich in flavonoids and saponins, in folk medicine is not surprising.

The n-butanol extract with 66.37±1.03% maximum reduction at 400mg/kg body weight indicated that the fruit of *H.thebaica*was known to posse's phytochemical constituent with antidiabetic properties.[17] The n-butanol fraction is devoid of saponins but contained carbohydrates, flavonoids, tannins and terpenoid. It was reported that aqueous extract of *Hyphaenethebaica* contains saponins, flavonoids and alkaloids.[18] In another report, *Hyphaenethebaic*a epicarp contained different flavonoids in its active water soluble fraction, such as tuteolin and chrysoerial which help in the

improvement of glucose and insulin tolerance.[19] Tuteolin inhibits alpha glucosidase and alpha-amylase activities resulting in the decrease in postprandial hyperglycaemia by reducing high blood glucose level.[19,20]

The fraction C was found to retain the highest hypoglycaemic activities (45.33±2.80%) among the seven sub-fractions. Researchers reported over 150 plant extracts and some of their active principle including flavonoids which are known to be used for the treatment of diabetes.[21] Fraction c_1 was very active; had maximum reduction of 60.51± 1.02% at 100mg/kg. phytochemical analysis showed it contains carbohydrates and high level of flavonoids. Five flavones glycosides were isolated and identified from doum fruit this include 7-0-βglucuronoidsapigen 7-0-βglucuronoid, luteolin-7-0 rutinoside and chrysoerial 7-0-rutinoside.[22] Doum leaves, four major flavoidal compound were identified as quercetin glucoside, Kaemferolrhamnoglucoside and dimethyloxylequercetin rhammoglucoside.[23] A mechanism proposing the reduction in hyperglyceamia was reported, that flavonoid, quercetin and ferulic acid have effect on pancreatic β cell leading to the secretion of more insulin in diabetic rats.[24]

The flavonoids in this extract may be responsible for the hypoglycaemic effect of *H. thebaica* fruit pulp.

REFERENCES

1. Burdkit, HM (Ed). Taxonomy of *H. thebaica* (L.)Mart. The Useful Plants of West Tropical Africa.(1st ed), Royal Botanical Gardens, Kew, England 1987: P 338.
2. Kamis, AB, Modu, S, Zanna, H, Oniyangi, TA. Preliminary biochemical and haematological effects of aqueous suspension of pulp of *Hyphaenethebaica* (L) mart in rats. Biokemistri 2003; 13: 1 – 7.
3. Kamis, A B., Modu, S, Markus, PY. Some biochemical effects of various doses of ethanolic pulp extract of *Hyphaenethebaica* .Nig Exper Appl Biol 2001: 33-36.
4. Modu, S, Kamis, AB, Markus, PY. Some biochemical effects of aqueous pulp extract of *Hyphaenethebaica (*L*)* Mart determination in rats'. Life Environ Sci 2000; 2/3: 139-143.
5. Helta, MH, Yasin, NZ, El Shaer, MA. Effects of *Hyphaenethebaica*on the spermatogenesis of male rats. The Egyp Med J Natl Res Centre 2005; 4:35 – 39
6. Irobi, ON, Adedayo, O. Antifungal activity of aqueous extract of dormant fruits Of *Hyphaenethebaica* (palmae). Pharm Biol 1999; 37: 144 – 117.
7. Zanna, H, Adeniji, S, Shehu, BB, Modu, S, Ishaq GM. Effects of aqueous suspension of the root of *Hypheanethebaica* (L.). Mart in some indicators of liver and kidney function in rats. J Pharmacol Toxicol 2008;3(4):330-33
8. Shehu, BB, Gidado, A, Buratai.LB. Hypoglycaemic effect of extract of *Hyphaenethebaica* (L) Mart fruit pulp in normal and alloxan induced diabetic rats. J Med Appl Biosc 2014; 6(1):6-15.
9. Shehu, BB, Gidado. A, Buratai.LB, Benisheikh AAG. Sub-acute toxicity studies of methanol extract of *Hyphaenethebaica* (L) Mart fruit pulp on normal Wister albino rats. Intl. *J of Res (IJR)* 2015; Volume 2,(6) Pp 460-467
10. Faraz, HSP, Kanner J. Phytochemicals of medicinal plants and their uses. *African* J Trad Med Plants 2003; 6(4); 122-126
11. Prince, PMS, Menon, VP. Hypoglycaemic and hypolipidaemic action of alcohol extract of *Tinosporacordifolia* roots in chemical induced diabetes in rats. Phytother Res 2003; 17:410 – 413.
12. Rhenry CC, Kirk KK. Performance of three blood glucose meters: Amer J Pharmacother 2000; 34(3): 317 – 321.
13. Treases, GE, Evans, MC. Textbook of Pharmacognosy, 13th ed. Bailliere, Tindall, London 1989: 135 – 149.
14. Richard, JPC (Ed). Natural products isolation. Methods in Biotechnology. Glaxo Welcome Research and Development. Stervenage, Herts, UK Human Press Totowa New Jersey 1984; Pp 343-363.
15. Dosumu, OO, Nwosu, FD, Nwogu, CD. Antimicrobial studies and phytochemical screening of extracts of *Hyphaenethebaica.* Trop Med 2006; 4:186-189.

16. Waterhouse, A. Saponins, a new cholesterol fighter, found in red wine.226th National Meeting of the American Chemical Society. New York, 2003:7-11.
17. Abdel-Rahman EA, El-Beltagi H, Fayed A. Comparative studies on the influence of *Juniperus. phoenicea* and *Hyphaenethebaica* as hypoglyceamic factors in diabetic rats. J Adv Food Scie 2011; 33 128-132.
18. Hsu, B, Coupar, LM, Ken, NG. Anti-oxidant activity of hot water extract from fruit of the Doum palm *Hyphaenethebaica*. Food Chem 2006; 98 (2): 317 – 328.
19. Salib, JY Micheal, HN, Eskande EF. Anti diabetic properties of flavonoids compounds isolated from *Hyphaenethebaica*epicarp on alloxan induced diabetic rats 2013; Pharmacog Res; 5: 22-29.
20. Abdul-Moneim M, Mustafa HN, Megahed MA., Agaibyi MH., Megazy GA, El-Daba M.A. The ameliorative potential of *Hyphaenethabaica*on Streptozotocin induced diabetic nephropathy. Folia Morphol 2015; 74(4): 447-457.
21. Chude, MA, Orisakwe, OG., Afome, OJ. Gamaniel, KS, Vongtau, OH, Obi, E. Hypoglycaemic effect of the aqueous extract of *Boerhaviadiffusa* leaves. Indian J Pharmacol 2001; 33:215-216
22. Amany, NH. Phytochemical investigation of the fruit of *Hyphaenethebaica* (L) Mart growing in Egypt family palmae. Thesis of masters; Natl Res Centre, GIZA Egypt. 1994; P 42
23. Omayama, AN E, Nahla, AA, Abdel-Nasser, BS, Mohammed, MA. Potential superoxide anion radical scavenging activity of Doum palm *Hyphaenethebaica*leaves extract. Nat Products 2008; 2(3):83 – 93.
24. Mehesh T, Menon PV. Quecetinalleviates Oxidative Stress in Streptozotocin induced diabetic rats. Phytother Res 2004; 18: 123-12
25. Graphpad Instant. GraphPad version 3.00 for 95, Graphpad software San Diego, California, U.S.A.2003. Available at www.graphad.com.