Research Article

www.ijrap.net

IN VITRO AND IN SILICO ANTI-DIABETIC ACTIVITY OF PHTHALIC ACID ISOLATED FROM *PHYLLANTHUS RHEEDII*

V. Sivajothi[1]*, Shruthi SD[2]

[1]Department of Pharmaceutical Chemistry, The Oxford College of Pharmacy, Bangalore, Karnataka, India

[2]Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore, Karnataka, India

Received on: 26/07/13 Revised on: 21/08/13 Accepted on: 31/10/13

***Corresponding author**

E-mail: writetojothi@yahoo.co.in

DOI: 10.7897/2277-4343.04623

Published by Moksha Publishing House. Website www.mokshaph.com

ABSTRACT

In folk remedies, the whole plant of *Phyllanthus rheedii* is used to treat diabetes. This study aimed to investigate the *in vitro* and *in silico* antidiabetic effects of isolated Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester from the chloroform extract of whole plant of *Phyllanthus rheedii* and AMP kinase activation property of the isolated compound. The Phthalic acid binds very efficiently within the active pocket of AMPK with the formation of 2 hydrogen bonds which is good when compared to orientation of standard drug Metformin. In *in vitro* antidiabetic evaluation of STZ treated chick embryo, the administration of isolated compound at a doses of 0.5 mg/egg and 1 mg/egg produced a significant reduction in the blood glucose levels in a dose dependant manner ($p < 0.01$). The blood glucose level of diabetic control was 244.2 ± 12.64 mg%, whereas it was 207.4 ± 2.43 mg% ($P < 0.001$) for isolated compound at dose of 0.5 mg/egg and 174.8 ± 2.410 mg% ($P < 0.001$) for dose of 1 mg/egg of the isolated compound. The significant glucose levels were reduced ($p < 0.01$) after administration of the compound.

Keywords: *Phyllanthus rheedii*, Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester, AMP kinase, chick embryo.

INTRODUCTION

Phyllanthus rheedii, a plant which belongs to the family of Euphorbiaceae is a slender branching erect herb. It has the calyx-lobes usually with white margined. It is found throughout India. It is used as an oriental folk medicine in diabetes mellitus[1]. The ethanol extract of *Phyllanthus rheedii* has a preventive effect on d-galactosamine induced hepatic damage in Wistar rats[2]. The alcohol extract of *Phyllanthus rheedii* showed a significant hepatoprotective and antiviral effects[3]. Cytotoxicity Screening was performed for the alcoholic extract of the whole plant of *Phyllanthus rheedii* [4]. Diabetes mellitus is a metabolic disorder. Chronic elevation of blood glucose level leads to retinopathy, neuropathy and peripheral vascular insufficiencies[5]. In olden days it was considered a disease of minor significance. In the 21st century, it is one of the main threats to human health. It is estimated that 300 million people will be affected by diabetes mellitus in the year 2025[6]. Increasing diabetes and obesity among the people in the world intensified search for new therapeutic treatment for diabetes mellitus[7]. AMP-activated protein kinase (AMPK) is an enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ)[8,9]. There are two isoforms of the catalytic subunit: AMPK α 1, which is widely distributed, and AMPK α 2, which is expressed in skeletal muscle, heart, and liver[10]. AMPK works as an intracellular fuel gauge that becomes activated by decreases in the ATP/ADP and phosphocreatine (PCr)/creatine ratios through mechanisms involving phosphorylation by one or more upstream AMPK kinases, allosteric activation, and a decrease in the inhibitory action of phosphatases[11]. The increase in AMPK activity stimulates the glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis[12]. The AMPK system is a regulator of energy balance at both the cellular and whole-body levels, once gets activated by low energy status effects a switch from ATP-consuming anabolic pathways to ATP-producing catabolic pathways. That is why it is now appearing to be the major target to treat type 2 diabetes[13]. The objective of the present study was to investigate the *in vitro* and in silico antidiabetic effects of isolated Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester from the whole plant of *Phyllanthus rheedii* and AMP kinase activation property of the isolated compound.

MATERIALS AND METHODS

Plant Material and Preparation of Plant Extract

The whole plant of *Phyllanthus rheedii* was collected near the Yercaud hills, India in the month of October and was authenticated by the Botanist, Botanical Survey of India, Coimbatore, India. A voucher specimen has been stored and maintainedin our laboratory (ET-30). The plants dried in shade and powdered. The powder was extracted with ethanol (95 % v/v) using Soxhlet apparatus. The extract was dried under reduced pressure and stored in a desiccator. The yield of extract was 3.5 % w/w thus obtained crude extract was used for phytochemical screening. The ethanol extract was chromatographed over silica gel 60–120 mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. Then the chloroform extract was used for isolation.

Phytochemical Screening

The crude extract was qualitatively examined for the presence of various phytochemical constituents using standard tests as described by Harborne[14].

Isolation of Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester

Column Chromatographic Separation of Chloroform Extract of *Phyllanthus rheedii*

The chloroform extract (4.5 g) was chromate graphed over silica gel 60 - 120 mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 15 ml portions and monitored on TLC with iodine vapor as a detector. The fractions that showed similar spots were combined. The fractions 144-166 eluted in petroleum ether: chloroform (40:60) gave a yellow residue on concentration and showed one major spot and two minor spots. Hence, this fraction was concentrated and freeze dried and re chromate graphed for further purification. The yellow residue (0.200 g) obtained in this fraction was further chromate graphed over silica gel 100 - 200 mesh of column length 75 cm and diameter 1.2 cm. Gradient elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 15 ml portions and monitored on TLC. The fractions 55 – 68 eluted in petroleum ether: chloroform (40:60) solvent system offered a yellow color semi solid and gave a single spot on TLC (Chloroform: petroleum ether 60:40) with R_f value of 0.477. It was re crystallized in methanol to obtain compound with yield 35 mg. This compound was subjected to physical and spectral studies for confirming the purity and characterization.

Effect of on STZ Treated Chick Embryo

Streptozotocin (STZ) was first reported to have a specific diabetogenic effect[15]. In an attempt to reduce the number of mammals used in drug research, the use of chick embryos found superior for predicting the effects of drugs[16-20]. Pancreas of chick embryo lies in duodenum that consists of a long U- shaped loop. It begins to develop rapidly during the 5th day of incubation and to acquire its definitive structure by 12th day of incubation. β cells secrete insulin from the 4th or 5th day of incubation[21]. The level of serum insulin in chick embryos increases gradually after the 12th day of incubation. The mechanism of induction of diabetes by STZ was not known.

Selection of Animals

Fertile eggs of White Leghorn chicks were obtained from poultry were incubated at 37.5 ± 0.2^{o}C at a relative humidity of about 65 %, turned automatically every hour.

Experimental Induction of Diabetes

Fertile eggs of White Leghorn chicks obtained from poultry were incubated at 37.5 ± 0.2^{o}C at a relative humidity of about 65 %, turned automatically every hour. For induction of diabetes STZ was dissolved in physiological saline and sterilized through a membrane filter. 300 µg/egg of STZ was injected into the albumen of fertile eggs on the 14th day of incubation.

Sample Collection

Large vitelline veins of eggs were selected and marked by pencil under the florescent lamb. Eggshell of a marked range of 10 x 5 mm was removed by electrical drill and a drop of water was filled to project clearly an artery. Whole blood was collected from a vein of egg by means of a tuberculin syringe with a 0.55 x 32 mm needle. The collected blood samples were immediately centrifuged at 2000 rpm for 15 minutes. The serum separated out was collected in fresh serum tubes and stored in refrigerator (2-4°C) after tightly capped until analysis.

Experimental Design

After the induction of diabetes the eggs were divided into seven groups of ten each. The eggs were divided into seven groups of ten each as detailed below.

Group I - Control eggs received the vehicle (saline injected into the albumen on 17th day)
Group II - Diabetic control received the vehicle (saline injected into the albumen on 17th day)
Group III - Diabetic control received isolated Compound (0.5 mg/egg injected into the albumen on 17th day)
Group IV - Diabetic control received isolated Compound (1 mg/egg injected into the albumen on 17th day)
Group V- Diabetic control received insulin (4 U/egg injected into the albumen on 17th day)

The blood sample was collected on the 17th day of incubation before and after 5min of injection of the compound and insulin and analyzed for blood glucose levels using strip method.

In Silico Docking Studies

Automated docking along with a graphical user interface, Auto Dock tools was utilized to generate grids, calculate dock score and evaluate the conformers of inhibitors bound in the active site of AMPK as targets for antidiabetic activity. A Lamarckian genetic algorithm method, implemented in the program Auto Dock 3.0, was employed to get docking and binding scores. The ligand molecules Phthalic acid and Metformin were designed and the structure was analysed using Chem Draw Ultra 6.0. The Pro drug server was used to minimise energy of drug compounds and 3D coordinates were prepared. The protein structure file (PDB ID: 1ZON) was taken from PDB (www.rcsb.org/pdb) and was edited by removing the heteroatoms and C terminal oxygen was added using Swiss PDB viewer. As per genetic algorithm all the torsions were allowed to rotate during docking. The grid map was centred at particular residues of the protein and was generated with Auto Grid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters.[22-25]

Statistical Analysis

All data were expressed as the means ± standard deviation (SD). Student's t- test was used to arrive at the statistically significant changes associated with various treatments. $p < 0.05$ was regarded as significant.

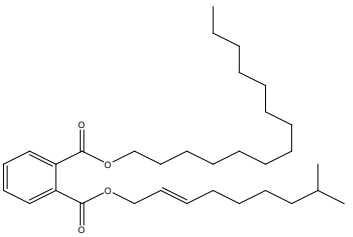

Figure 1: Structure of Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester

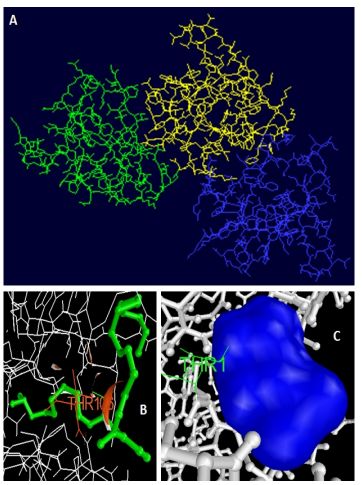

Figure 2: (a) Structure of AMPK as seen in swiss PDB viewer. (b) Orientation of phthalic acid in the active pocket of AMPK. (c) Enfolding of metformin in active pocket

Table 1: Effect of Isolated Compound Phthalic Acid on Stz Treated Chick Embryo

Design of Treatment	Dose (mg/kg)	Blood glucose (mg%)
Control	-	156.55 ± 2.286
Saline control	-	151 ± 2.15
Diabetic control	-	244.2 ± 2.64**
Compound Phthalic acid	0.5 mg/egg	207.4 ± 2.43*
Compound Phthalic acid	1 mg/egg	174.8 ± 2.410*
Insulin	4 U/egg	157.6 ± 1.986*

Values represent mean (± SEM). (n = 6), *P < 0.001 Vs diabetic control, **P < 0.01 Vs control.
Data were analyzed by one way ANOVA followed by Tukey multiple comparison analysis.

Table 2: Molecular Docking Results of AMPK

Compounds	Binding energy	Docking energy	Inhibitory constant	H-bonds	Bond formation
PA	7.31	12.83	-	2	1B::DRG1:OAH::AMPK:A:THR106:OG1 1B::DRG1:OAZ::AMPK:A:THR106:HG1
MF	-5.54	-5.54	8.69e-005	1	MF::DRG1:HAB::AMPK:A:THR106:OG1

PA - Phthalic acid, MF - Metformin

RESULTS AND DISCUSSION

The ethanolic extract was extracted from the whole plant of *Phyllanthus rheedii* and the preliminary phytochemical screening of crude extract indicates the presence of flavonoids, glycosides, triterpenoids, saponins and tannins. The crude extract was subjected to column chromatography to get Phthalic acid 1-(8-methyl-non-2-enyl) ester 2-tetradecyl ester (Figure 1). The IR (KBr) spectrum showed absorption for a carbonyl group at 1710 cm^{-1}, unsaturation at 1618 cm^{-1} and an isopropyl unit at 1457 cm^{-1} and 1377 cm^{-1}. ^{1}H-NMR (DMSO) spectrum exhibits signals at δ 7.29 (m, 1H), 7.58 (m, 1H) and δ 7.76 (m, 2H) indicating the presence of a disubstituted aromatic ring. The signals at δ 4.30 (m, 2H) and 3.50 (m, 2H) were assigned to two oxy methylene groups of an ester moiety. In addition to this signal shift exhibits signals at δ 2.84, δ 2.1-2.3, 1.3 to 1.7 and 0.9 for methine, methylene and methyl groups indicating the presence of long chain hydrocarbon moiety in the molecule.^{13}C NMR exhibited a signal at δ 165.89 (characteristic of phthalic acid ester carbonyl), eight signals between δ 130.58 to 126.07 for a benzene ring and a double bond in the long chain part, δ 66.42 oxy methylene groups and the rest of the methyl and methylene groups appeared between δ 36.85 to 12.24. In MASS spectrum, the molecular ion peak was observed at m/z 428 which indicated the molecular weight of the compound. The melting point of the compound was recorded on electro thermal melting

point apparatus and observed melting point was found to be 182°C. Administration of isolated compound at a dose of 0.5 mg/egg and 1 mg/egg produced a significant reduction in the blood glucose levels in a dose dependant manner ($p < 0.01$). The blood glucose level of diabetic control was 244.2 ± 12.64 mg%, whereas it was 207.4 ± 2.43 mg% ($p < 0.001$) for compound 0.5 mg/egg and 174.8 ± 2.410 mg% ($p < 0.001$) for 1 mg/ egg of the isolated compound. The significant glucose levels were reduced ($p < 0.01$) after administration of the compound as shown in Table 1. The results obtained in *in silico* studies are in correlation with the *in vitro* studies. The Phthalic acid binds very efficiently within the active pocket of AMPK with the formation of 2 hydrogen bonds which is good when compared to orientation of standard drug (Figure 2). But the binding energy required is more than standard Metformin as shown in Table 2. Studies related to improving its structure where it requires less binding energy to dock are in progress. Hence, Phthalic acid can be considered as effective treatment against diabetes.

CONCLUSION

Phthalic Acid 1-(8-Methyl-Non-2-Enyl) Ester 2-Tetradecyl Ester isolated from the chloroform extract of the whole plant of *Phyllanthus rheedii* showed a remarkable *in vitro* and *in silico* antidiabetic activity. It has demonstrated a dose dependant efficacy. Detail study is required on its effects *in vivo* and also to evaluate its bio-safety and clinical potentials. However, experimental validation of the predicted compound in this direction is needed.

REFERENCES

1. Rajan S, Sethuraman M, Mukherjee PK. Ethno biology of the Nilgiri Hills. India Phytother Res 2002; 16: 98-116.
2. Suresh V, Asha VV. Preventive effect of ethanol extract of *Phyllanthus rheedii* Wight on D-galactosamine induced hepatic damage in Wistar rats. Journal of Ethno pharmacology 2008; 116: 447-453. http://dx.doi.org/10.1016/j.jep.2007.12.011 PMid:18243611
3. Suresh V, Asha VV. Hepatoprotective and antiviral activities of *Phyllanthus rheedii* Wight. A plant used in Traditional Medicine. Proceedings of 20th Kerala Science Congress; 2008. p. 561-564.
4. Sivajothi V, Leelaprakash G. Cytotoxicity Screening of alcoholic Extract of the Whole Plant of *Phyllanthus rheedii*. Res J chem Environ 2010; 14: 81-2. http://www.shankargargh.net/ chemistry_back_issue/chem_2010_3/16.pdf
5. Joe MC, Arshag DM. A rational approach to drug therapy of type 2 Diabetes Mellitus. Drugs 2000; 60: 95–113. http://dx.doi.org/10.2165/00003495-200060010-00006
6. Pradeepa R, Deepa R, Mohan V. Epidemiology of diabetes in India--current perspective and future projections. J Indian Med Assoc 2002; 100: 144-8. http://www.ncbi.nlm.nih.gov/pubmed/12408271. PMid:12408271
7. Kumar VR, Kumar V, Kothari A, Mital A, Ramachandran U. Emerging targets for diabetes. Current Science 2005; 88: 241-249. http://www.currentscience.ac.in/Downloads/article_id_088_02_0241_0249_0.pdf
8. Hardie DG, Carling D, Carlson M. The AMP activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 1998; 67: 821–855. http://www.ncbi.nlm.nih.gov/pubmed/9759505. http://dx.doi.org/10.1146/annurev.biochem.67.1.821 PMid:9759505
9. Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA. Dealing with energy demand: the AMP-activated protein kinase. Trends Bio chem Sci 1999; 24: 22–25. http://www.ncbi.nlm.nih.gov/pubmed/10087918. http://dx.doi.org/10.1016/S0968-0004(98)01340-1
10. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, The T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE. Mammalian AMP-activated protein kinase subfamily. J Biol Chem 1996; 271: 611–614. http://dx.doi.org/10.1074/jbc.271.2.611 PMid:8557660
11. Hardie DG, Carling D. The AMP-activated protein kinase-fuel gauge of the mammalian cell. Eur J Biochem 1997; 246: 259–273. http://dx.doi.org/10.1111/j.1432-1033.1997.00259.x PMid:9208914
12. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D. Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. EMBO J 1998; 17: 1688–1699. http://dx.doi.org/10.1093/emboj/17.6.1688 PMid:9501090 PMCid:PMC1170516
13. Hardie DG. AMP-activated protein kinase as a drug target. Annu Rev Pharmacol Toxicol 2007; 47: 185-210. http://dx.doi.org/10.1146/annurev.pharmtox.47.120505.105304 PMid:16879084
14. Harborne JB. Phytochemical methods. London: Chapman and Hall Ltd; 1973. p. 49-188.
15. Rakieten N, Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin. Cancer Chemother Rep 1963; 29: 91-98. http://www.ncbi.nlm.nih.gov/pubmed/13990586
16. Sugiyama T, Miyazaki H, Saito K, Shimada H, Miyamoto KI. Chick embryos as an alternative experimental animal for cardiovascular investigations: Stable recording of electrocardiogram of chick embryos in ovo on the 16th day of incubation. Toxicol Appl Pharmacol 1996; 138: 262–267. http://dx.doi.org/10.1006/taap.1996.0124
17. Yoshiyama Y, Sugiyama T, Miyazaki H, Kanke M, Shimada H. Toxic Interactions between Disopyramide and Propranolol in Chick Embryos. Biol Pharm Bull 1997; 20: 1310-1312. http://dx.doi.org/10.1248/bpb.20.1310 PMid:9448111
18. Yoshiyama Y, Sugiyama T, Kanke M, Tsuchimoto K. Influence of the Light Schedule on the Toxic Interaction between Propranolol and Disopyramide in Chick Embryos. Biol Pharm Bull 2001; 24: 429-431. http://dx.doi.org/10.1248/bpb.24.429 PMid:11305609
19. Yoshiyama Y, Sugiyama T, Tsuchimoto K, Kanke M. Drug-drug interaction between anti arrhythmic drugs in chick embryos. Biol pharm Bull 2004; 27: 128-130.http://dx.doi.org/10.1248/bpb.27.128
20. Yoshiyama Y, Kanke M. Toxic Interactions between Fluconazole and Disopyramide in Chick Embryos. Biol Pharm Bull 2005; 28: 151-153.http://dx.doi.org/10.1248/bpb.28.151
21. Prezybylski RJ. Cyto differentiation of the chick pancreas, Gen Comp Endocr 1967; 8: 115—128. http://dx.doi.org/10.1016/0016-6480(67)90120-7
22. Ghose AK, Crippen GM. Atomic physicochemical parameters for three dimensional-structure-directed quantitative structure activity relationships. Modelling dispersive and hydrophobic interactions. J Chem Inf Comput Sci 1987; 27: 21–35. http://dx.doi.org/10.1021/ci00053a005 PMid:355850
23. Binkowski TA, Naghibzadeg S, Liang J. CASTp computed atlas of surface topography of proteins. Nucleic Acid Res 2003; 31: 3352–3355. http://dx.doi.org/10.1093/nar/gkg512 PMid:12824325 PMCid:PMC168919
24. Vidya SM, Krishna V, Manjunatha BK, Rajesh KP, Bharath BR, Manjunatha H. Antibacterial and molecular docking studies of entagenic acid, a bioactive principle from seed kernel of *Entada pursaetha*. DC. Med Chem Res 2012; 21: 3195-3203. http://dx.doi.org/10.1007/s00044-011-9614-4
25. SD Shruthi, S Padmalatha Rai, YL Ramachandra. Isolation, characterization, antibacterial, anti helminthic, and in silico studies of polyprenol from *Kirganelia reticulata* Baill, 2012, Med Chem Res 2013; 22: 2938–2945.. http://dx.doi.org/10.1007/s00044-012-0295-4

Cite this article as:
V. Sivajothi, Shruthi SD. In vitro and in silico anti-diabetic activity of phthalic acid isolated from Phyllanthus rheedii. Int. J. Res. Ayurveda Pharm. 2013;4(6):889-892 http://dx.doi.org/10.7897/2277-4343.04623

Source of support: Nil, Conflict of interest: None Declared