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Diosgenin From *Dioscorea Alata*: Extraction and Potential Effects on Enzymes Related to Metabolic Syndrome

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Abstract

The exponential increase in the prevalence of Metabolic Syndrome in recent times due to ever growing urbanization has led to the evaluation of bio actives extracted from botanical sources to ameliorate this effect. One such phytochemical, diosgenin, a steroidal saponin is known to be present richly in *Dioscoreaalata* L. (Purple yam, Family Dioscoreaceae). The objective of the study was to determine the best solvent system, method for extraction of diosgenin and evaluation of its role in inhibiting enzymes involved in inflammation, diabetes and obesity. IC_{50} values for *in vitro* anti-inflammatory activities were evaluated by heat induced protein denaturation ($IC_{50\ aspirin}$= 1.76 µg/ml, $IC_{50\ AHS}$= 0.16 µg/ml). The IC_{50} values for *in vitro* inhibition of anti-diabetic activities were evaluated by the following assays: α-amylase inhibition ($IC_{50\ acarbose}$= 445.7 µg/ml, $IC_{50\ AHS}$=273.7µg/ml) and pancreatic lipase ($IC_{50\ orlistat}$=629.08µg/ml, $IC_{50\ AHS}$=490.36µg/ml).From the glucose diffusion assay, it is evident that diosgenin helps in delaying the glucose diffusion for up to 4h, thereby providing sufficient time for the system to eliminate glucose and prevent its uptake into the cells of the body. From the results obtained from these assays it suffices to predict that diosgenin from *D. alata* is a promising treatment for the regulation of diseases related to the metabolic syndrome and functions mainly by altering the absorption of glucose and inhibiting key metabolic enzymes.

Keywords

Acid hydrolysis, diosgenin, *Dioscorea,* anti-inflammatory, anti-diabetic, glucose diffusion

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INTRODUCTON

The term “Metabolic syndrome” first coined by Gerald Reaven[1] includes pathologies such as obesity, glucose intolerance, dyslipidemia, and hypertension that are inextricably linked to diabetes mellitus and cardiovascular disease. Its occurrence has risen to epidemic levels with diabetic cases alone projected to increase to 552 million by 2030[2]. The side effects associated with the long-term use of several drugs for the treatment of pathologies associated with metabolic syndrome is an impetus to identify natural products able to perform similar ameliorating roles.

One such species is *Dioscorea alata* L. (yam), a genus of over 600 species of herbaceous perennial plants that produce starchy, edible tuberous roots that are an important source of food in tropical regions worldwide. Yams however must be safely consumed after suitable cooking processes because raw yams contain various natural compounds, including phenols, tannins, hydrogen cyanide, oxalate, amylase inhibitor and trypsin inhibitor activities that can cause illness or lead to nutritional deficiencies. The *D.alata*s pecies are known to be rich in saponins, a diverse group of compounds characterized by the presence of triterpene or steroid aglycone and one or more sugar chains. The recent demand for natural products in food, cosmetics, and pharmaceutical sectors coupled to the presence of surfactant properties and the increasing evidence in anticancer and anticholesterol activity has led to the emergence of saponins as commercially significant compounds[3].Saponins are used in the pharmaceutical industry as raw materials for the synthesis of immunological adjuvants [4],production of hormones [5] and treatment of various cognitive impairment [6]. The natural surfactant properties of saponins is utilized in personal care sectors producing shampoos, shower gels, hair conditioners, liquid soaps, baby care products and toothpastes [7,8]. Additionally, they are used as bioactive ingredients in cosmetic formulations for anti-aging [9] and anti-acne [10] products.

Steroidal drugs are the costliest group of drugs used throughout the world today. Diosgenin [(25R)-spirost-5-en-3b-ol], often prescribed as medicine [11] is the most pharmaceutically important steroidal sapogenin present in *D.alata*. It has been used extensively in traditional medicine leading to series of preclinical and mechanistic studies in order to fully understand and confirm its role in a variety of pathologies including diabetes, hyperlipidemia, cancer, cardiovascular disease, oxidative stress and inflammation [12,13,14]. It is a precursor of sex hormones(progesterone), corticosteroids (corticosone) and contraceptives [15,16]. Dioscin derived from Diosgenin is known to induce apoptosis in HeLa cells through caspase-9 and caspase-3pathway [17]. It causes an inhibition of growth of fibroblast-like synoviocytes in human rheumatoid arthritis with apoptosis induction associated withcyclooxygenase-2 up-regulation [18]. Cholesterol-lowering ability of diosgenin in human clinical trials is related to its ability to inhibit the absorption of cholesterol from the small intestine, or the re-absorption of bile acids [19].

The complex nature and diversity of saponins has led to the development of new approaches to overcome the challenge. The extraction process involves the use of extraction solvent under suitable extraction conditions (such as temperature, time and pH). This research paper highlights the acid hydrolysis method of extraction and concentration of diosgenin from *D. alata.*

MATERIALS AND METHODS

Plant material: The experimental plant material used for extraction and characterization studies of the active secondary metabolite, Diosgenin fromyam (*Dioscoreaalata* L.) were obtained from tubers that were purchased via random sampling from the local market in Sultanpalya, R.T. Nagar, Bengaluru. The procured samples were manually scoured, rinsed thoroughly in running tap water for 15 min, blotted dried, cut into thin slices, dehydrated under direct sunlight and finely powdered using a laboratory grinder.

Fluorescence analysis of powder: A pinch of the fine powder was taken on a slide and added with a few drops of different chemical reagents. The slide was placed inside the UV viewer chamber and viewed in visible light and short ultraviolet radiations (254 nm). The characteristic colour produced due to different reactions were observed and recorded as per standard methods [20,21].

Preliminary phytochemical investigation: Qualitative phyto-chemical examinations were carried out for all the extracts; water (WE), methanol (ME), ethanol (EE), methanol: water (1:1, MWE) and ethanol: water (1:1, EWE)as per the standard methodology[22,23] to confirm the presence of phyto constituents such as carbohydrates, alkaloids, flavonoids, glycosides, phenols, saponins, tannins and terpenoids.

Extraction of Diosgenin by Acid Hydrolysis: The process of extraction of diosgenin from *D. alata* was done according to method of Drapeau et al., [24]. 5 g of the dried tuber powder was weighed, subjected to refluxing for a period of 8 h in 20% H_2SO_4 and 150 ml 70% isopropanol. This was followed by filtration and the resultant filtrate washed thrice with 50 ml hexane. The three batches of hexane extracts obtained were

pooled and washed with 5% NaOH, followed by rinsing with distilled water. The extracts were allowed to evaporate to dryness and then re-dissolved in 10 ml of ethanol and stored at 4˚C until further analysis. An aliquote was used for quantitative estimation of diosgenin.

Estimation of Diosgenin content

***Standard curve for Diosgenin*:** Aliquots of 0.0, 0.2, 0.4,0.6, 0.8, 1.0 ml of working standard of diosgenin in methanol (100 µg/ml) were taken for the construction of the standard curve for diosgenin[25].The respective solutions were made up to 2 ml using ethyl acetate and then processed by treatment with 1 ml each of the color developing reagents 1 (Conc. H_2SO_4: ethyl acetate, 1:1 ratio) and 2 (0.5ml p-anis aldehyde in 99.5 ml of ethyl acetate)[26].

***Estimation of Diosgenin in the samples*:**

The estimation of diosgenin was performed as mentioned by Chapagain and Weisman [27] with various modifications.

For non-diosgenin extracted samples**:**10 mg of the dried yam tuber powder was weighed and homogenized in 5 ml of methanol. 0.2 ml of this solution was taken in a test tube and the volume made up to 2 ml using ethyl acetate and processed with 1 ml of the Colour Reagents 1 and 2. The test tubes were capped and incubated at 60 ˚C for 10 min. After the test tubes were cooled, 0.5 ml of distilled water was added to each test tube. 2 ml of ethyl acetate was used as blank and processed in the similar manner. Calibration curve was obtained by using diosgenin as the standard (100µg/ml working standard).

For diosgenin extracted samples: The estimation of diosgenin in the extracted crude samples were processed slightly different than the non-extracted samples. About 0.1 ml of the ethanolic extracts were taken into a test tube and the volume made up to 2 ml with ethyl acetate. To this 1 ml of 5 % L-ascorbic acid solution was added, followed by the addition of 0.5 ml of Color Reagents 1 and 2. The test tubes were then processed as previously mentioned. Calibration curve was obtained by using diosgenin as the standard (100µg/ml) and ethyl acetate as blank.

***In vitro* analysis of anti-inflammatory activity**

***Inhibition of heat induced protein denaturation*:** *In vitro* anti-inflammatory activity of *D. alata*was studied by measuring its ability to inhibit heat induced albumin denaturation [28,29]. The reaction mixture comprised of the test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 ºC for 20 min. The turbidity was measured at 660 nm after cooling. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated using Eq. 1.

$$\% \, Inhibition = \frac{(OD \, of \, control - OD \, of \, sample)}{OD \, of \, control} \, x \, 100 \quad \text{---------- (Eq. 1)}$$

***In vitro* evaluation of anti-diabetic activity**

***α-Amylase inhibition assay*:** Pancreatic α-amylase inhibition assay was executed as described by Toma et al., [30].10 µl of the respective samples or acarbose at different concentrations (100-500 µg/ml) were incubated with 75 µl of porcine pancreatic amylase (3 U/ml) in phosphate buffer (100 mM, pH 6.8) at 37 °C for 10 min. Thereafter, 250 µl of0.1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was added to the reaction mixture and incubated at 37° C for10 min. The reaction was arrested by the addition of 1 mldinitro salicylic acid reagent (DNS) to the mixture and incubated for 10 min in a boiling water bath for colour development. The colour produced was then stabilized using 250 µl of40% potassium sodium tartarate. To this, 2 ml of phosphate buffer was added, and the absorbance was read at 540 nm using a spectrophotometer. Phosphate buffer with DNS was used as a blank. Acarbose was used as positive control. The percentage inhibition of α-amylasewas calculated using Eq. 1.

***Pancreatic lipase inhibition assay*:** This assay was performed as described by Bustanji et al., [31]. The enzyme solution was prepared immediately before use by suspending crude porcine pancreatic lipase powder in 0.1 M phosphate buffer (pH 7.6) (100 U/ml). The solution was then centrifuged at 2000 rpm for 10 min and the clear supernatant was recovered. Triolein (1% v/v) was used as the substrate for pancreatic lipase. The yam extracts (100-500 µg/ml) were preincubated with 200 µl of enzyme solution for 5 min at 37 °C, before the addition of 800 µl triolein substrate solution. The absorbance was measured at 450 nm against blank using denatured enzyme in an ELISA reader. The denatured enzyme was prepared by boiling the enzyme solution for 5 min. Orlistat was used as a reference drug. The extract was dissolved in DMSO at a final concentration not exceeding 1% (v/v).

The activity of the negative control was checked in the presence and absence of the inhibitor. The % inhibition was calculated according to the formula (Eq. 1).

***In vitro* glucose diffusion inhibitory assay:** To study *in vitro* effects of the extracted diosgenin on the movement of glucose, a simple model system was adapted [32]. This model involved the use of dialysis tubes that were sealed containing 15 ml of 0.15M glucose and NaCl solution. The amount of glucose in the external solution was measured. This was used as the control. The experimental model contained a sealed dialysis tube into which 20 µl of plant extract in 2 ml of 0.5 % carboxy methyl cellulose (CMC) and 2 ml NaCl (0.15M) containing D-glucose (0.22M) was added. The tubes were sealed at either end and placed in a beaker containing 100 ml NaCl solution (0.15M). These beakers were then placed on a magnetic stirrer and maintained at room temperature. The glucose movement to the external solution was measured at given time intervals of 1h, 2h, 4h, 6h, and 27h.

Statistical analysis: Data of in vitro assays recorded were analyzed using Microsoft Excel to determine IC_{50}. One-way analysis of variance (ANOVA) were conducted and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

The utilization of distinctive substances particularly phytochemical mediators for the treatment of symptoms of metabolic syndrome has significantly picked up in recent years. One such bioactive is diosgenin, a steroidal sapogenin. The current research paper focusses on the method used for extraction of diosgenin from *Dioscorea alata* tubers, followed by evaluation of its anti-diabetic, anti-inflammatory and anti-obesity properties.

Preliminary investigation of the *D. alata*: The fine powder was mixed with different solvents and reagents and observed under visible and UV light (254nm). The colour data are presented in Table 1.

Table 1: Fluorescent analysis of tuber powder of *D. alata* with different reagents

Sl No.	Experiment	Visible/Day light	UV light (254 nm)
1	Powder+ distilled water	Brown	Purple
2	Powder+ 5% iodine	Dark blue	Black
3	Powder+picric acid	Yellow	Brown
4	Powder+Conc.H_2SO_4	Blackish brown	Fluorescent green
5	Powder+ Conc. HNO_3	Brown	Reddish brown
6	Powder+ Conc. HCl	Pale brown	Dark brown
7	Powder+1N NaOH	Brown	Brown
8	Powder+ Acetic acid	Brown	Fluorescent green
9	Powder+ Ferric chloride	Yellow	Fluorescent green
12	Powder+ Benzene	Brown	Purple
13	Powder+ Petroleum ether	Brown	Dark green
14	Powder+ Acetone	Brown	Purple
15	Powder+ Chloroform	Brown	Fluorescent green
16	Powder+ Methanol	Brown	Fluorescent yellow
17	Powder+ Ethanol	Brown	Fluorescent green

Phytochemicals are defined as secondary metabolites produced by majority of plants possessing medicinal uses. The phytochemical screening of tubers of *Dioscoreaalata* were analyzed in water (WE), methanol (ME), ethanol (EE), methanol:water (1:1, MWE) and ethanol:water (1:1, EWE) solvents respectively. The analysis of the phytochemical results revealed the presence of saponins, flavonoids, terpenoids, glycosides, phenols, alkaloids, terpenoids and glycosides in varying concentrations (Table 2). From the performed phytochemical screening it is evident that water is the best solvent for extraction of bioactives from yam and was hence used for further assays.

Table 2: Phytochemical investigation of *Dioscorea alata*

Phytochemical Analyzed	Tests performed	WE	ME	MWE (1:1)	EE	EWE (1:1)
Carbohydrates	Molisch's	+	+ +	+ + +	+	+ + +
	Benedict's	+ + +	–	+ +	-	+ +
	Fehling's	+ + +	–	+ +	-	+ +
Alkaloids	Mayer's	+ +	+ + +	-	+ +	+
	Wagner's	+ + +	–	+ +	+	+ +
	Dragendroff's	–	–	–	+ + +	+ +
Glycosides	Keller-Killiani's	+ + +	+ +	+	-	-
Saponins	Foam Test	+ + +	–	+ +	-	+
Phytosterols	Salkowski's	+ + +	+	+ +	-	-
Terpenoids	Salkowski's	+ +	+ + +	+	+	+ + +
Phenols	Ferric chloride Test	–	–	–	-	-
Flavonoids	Lead Acetate Test	+ + +	+	+ +	+ + +	-
Proteins & Amino Acids	Xanthoproteic Test	+ + +	+	+ +	-	+ + +
	Ninhydrin Test	+ + +	–	+ +	-	+ +

- indicates absence, + denotes average, ++ means abundance of phytochemicals.

Extraction and estimation of Diosgenin: The extraction procedures of the samples differ because of their properties on interaction with aqueous medium. Acid hydrolysis of *D. alata* samples was found to concentrate diosgenin about 12-fold. The amount of diosgenin in the extracted sample was found to be 1.35g/100 g. Apart from *D. alata,* there are various other Dioscorea species which contain varying concentration of Diosgenin. Diosgenin is absent in *D. Opposite* and *Heterosmilax japonica.* The former is used as a staple source of starch while the latter is commonly used in folk medicine. Various other species like *D. septemloba, D. collettii,* contain the diosgenin compound but the content is relatively small. Most of these plants are wide spread in the Asiatic region but majorly in China and thus a hub for diosgenin harvest [25].

Evaluation of anti-inflammatory activity: Inflammation involves the activity of a number of mediators such as cytokines, neutrophil derived free radical, nitric oxide (NO), prostaglandins and reactive oxygen species (ROS). An excessive production can lead to injury of the tissues by peroxidation of the lipid membrane and macromolecular damage; thus, leading to inflammatory diseases and pathogenesis.

Denaturation of proteins refers to the loss of secondary and tertiary structure of proteins in the presence of intrinsic and extrinsic stressful conditions. Denatured proteins are capable of instigating inflammation. This is one of the parameters investigated for understanding the potential of plant extract in inflammation. The IC_{50} values for standard aspirin and extracted diosgenin was found to be 1.76 µg/ml and 0.16 µg/ml respectively (Table 3). These values indicate that extracted diosgenin has optimal inhibitory activity when compared to aspirin. Yam is a lesser researched compound for its anti-inflammatory activity, the compound that possess the inhibitory properties is not well evaluated.

Evaluation of anti-diabetic activity: According to several *in vitro* and *in vivo* studies, diosgenin is known possesses protective benefits against metabolic diseases such as diabetes and obesity [33-37], metabolic syndrome [38], and hypercholesterolemia [39,40]. Diosgenin was found to reduce the plasma and triglyceride level in obese mice [41]. Antiatherogenic behavior of diosgenin is due to its ability to reduce intestinal cholesterol absorption and by suppression of MiR-19b induced downregulation of ATP-binding cassette transporter A1 in macrophages [42].

The enzyme α-amylase is responsible for converting polysaccharides into absorbable forms such as monosaccharides. Inhibiting this enzyme slows down the uptake of carbohydrate into blood circulation, a condition needed for hyperglycemic patients.

Maximum inhibition of 56.6% at 500 µg/ml was encountered by Acarbose, an inhibitor for pancreatic α-amylase, while extracted diosgenin exhibited 70.72% at the same concentration. The extracted diosgenin and Acarbose showed IC_{50} concentration of 273.7 and 445.7µg/ml respectively (Table 3). The IC_{50} values were calculated with the help of equation 1and documented in Table 3. The lower IC_{50} values of diosgenin extracted from yam is suggestive that it has a better inhibitory action.

The enzyme pancreatic lipase is involved in the hydrolytic conversion of dietary triacylglycerol to 2-monoacylglycerol and fatty acids prior to absorption. This conversion followed a dose-dependent manner of inhibition (Table 3). Maximum inhibition of 44.2% at 500 µg/ml was encountered by Orlistat, a pancreatic lipase inhibitor, while extracted diosgenin exhibited 41.4% at the same concentration. The IC_{50} values for Orlistat and diosgenin was found to be 629.08µg/ml and 490.36µg/ml respectively.

Table 3: Evaluation of *in vitro* anti-inflammatory and anti-diabetic activities by control drug and Diosgenin extracted from *D. alata*.

Conc (µg/ml)	% Inhibition					
	Protein denaturation		α -amylase		Pancreatic lipase	
	Aspirin	Test	Acarbose	Test	Orlistat	Test
100	8.2± 0.6	43.2±4.03	8.81 ± 0.3	28.8 ± 0.6	16.2 ± 3.3	13.2 ± 1.1
200	10.0± 0.8	51.0 ± 2.12	20.0± 1.3	48.8±4.2	36.3 ±7.8	29.6 ± 3.0
300	16.5± 1.1	73.6 ± 2.36	34.4 ± 2.7	55.5 ±7.6	40.0 ± 10.3	36.7 ± 4.6
400	81.3 ± 7.5	75.5±4.09	44.4 ± 4.8	58.8 ±5.1	35.8 ± 9.2	40.1 ± 6.7
500	92.1 ± 6.2	80.1±4.03	56.6 ± 5.9	70.72± 6.8	44.2 ± 5.9	41.4 ± 5.8
IC_{50}	**1.76**	**0.16**	**445.7**	**273.7**	**629.08**	**490.36**

Glucose diffusion test: To assess the anti-diabetic effect of diosgenin extracted from *D. alata*, the glucose diffusion test was performed. This resulted in varying levels of glucose from the 1hup to 4h, followed by a significant increase at 6 h and 27 h. The glucose concentrations for each time regime is summarized (Table 4, Fig 1) demonstrating the ability of diosgenin to delay glucose diffusion up to 4h. This enables the body to eliminate glucose successfully with reduced cellular uptake, an important need for obese or diabetic patients. Under such circumstances, glucose diffusion can be delayed by the employment of diosgenin, which can aid in glucose levels to be maintained. Similar results were obtained with diosgenin extracted from *Trigonellafoenumgraecum* seeds [33].

Table 4: Effect of *D.alata* extract on the movement of glucose out of dialysis tube over 27 h incubation period.

Samples	1h	2h	4h	6h	27h
Control	169.5 ± 0.007	28 ± 0.007	35 ± 0.014	16.4 ± 0.010	230 ± 0.004
D. alata	40.0 ± 0.004	14 ± 0.014	63.5 ± 0.030	110.7 ±0.045	838.4 ± 0.081

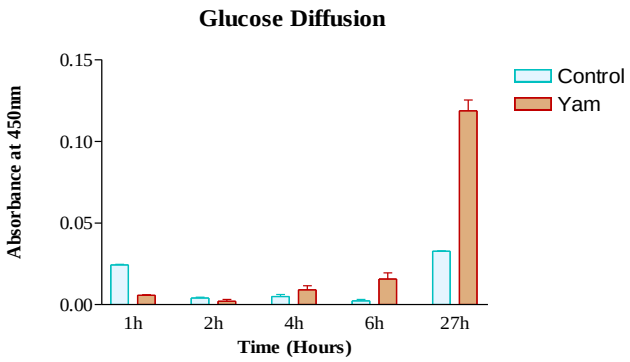

Fig 1: Effect of *D.alata* extracts on the movement of glucose out of dialysis tube over 27 h incubation period.

CONCLUSION

The evidence thus reported in this paper and the available literature on diosgenin supports the idea that the compound exerts beneficial effects on several physiologic markers relevant to metabolic syndrome. Diosgenin can therefore exerts an influence on the liver, pancreas, skeletal muscle and adipose tissue with the aim of restoring insulin sensitivity, glucose homeostasis and normal blood lipid profile. The treatment for Polycystic Ovary Syndrome (PCOS) and diabetes commonly involves the use of a biguanide called Metformin, that interrupts carbohydrate uptake. From our work as well as from previous studies, it is known that diosgenin brings about a similar effect. The various side effects of Metformin chiefly the irritation of the GI region can be eliminated by the use of diosgenin. Thus, diosgenin from *D. alata* can be considered a potential treatment for PCOS, hyperglycemia and obesity given its range of activities. Another distnctive feature is the structural similarity of diosgenin to estrogen, which is not observed in metformin. Thus, it suffices to say that diosgenin possess a 'Triple Effect' wherein it aids in regulating menstrual cycles, maintenance of lipid and blood glucose levels, which are distictive symptoms of an individual diagnosed with PCOS.

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