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Research Article

Phenotype Analysis as Plant Selection Strategy in Drug Discovery from Plants for Obesity

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Abstract: Introduction: It is conceivable that chemical constituents in plants are linked through genomics and proteomics to observable phenotypes such as plant height, shape of leaf, colour of leaves, flowers among others. Mathematical models including some of these phenotypes may be used to predict plants with potential activity against a particular disease. This hypothesis was tested, with obesity as target. **Methodology**: Retrieved database of 495 plants (48 active and 447 not active against obesity) was used to predict the utility of two phenotypes: height of plant (a continuous variable) and shape of leaf (a categorical variable) for the antiobesity activity using Receiver operator characteristic curve (ROC) analysis and Likelihood ratio respectively at pre-specified cutoff values. A single model combining the two phenotypes was developed. Aqueous leaf extract of 5 plants (based on the model), and five other plants (randomly selected) were screened in wet-lab experiment using high fat diet-induced obese rats. **Results**: Both height of plant (less than 0.30 m) and shape of leaf (simple leaf) were found to be predictive of the antiobesity pharmacological activity. On screening, all five plants (100%) selected using the model were correctly predicted to have anti-obesity activity, while only 3 out of 5 (60%) were found to possess weight reducing property using random selection. **Conclusion**: Mathematical modelling could be an effective predictor of pharmacological activity in plants but model refinement with more criteria may be required.
Keywords: model, obesity, phenotype, plant.

Introduction

Up to 25% of current conventional drugs, including many agents effective against obesity and diabetes, are derived from higher plants (Gurib-Fakim, 2006). A good example is metformin, the most frequently prescribed antidiabetic agent, which is derived from galanga. Moreover, more than half of People from developing countries use traditional medicine, such as plants for managing their ailments including obesity and diabetes (James *et al.,* 2018). The traditional methods for selecting plants for the testing of bioactivity is by the use of ethnobotanical information. However, it seems that this method has limited utility in some instances such as the exploration of agents effective against certain disease conditions such as cancer or metabolic disorders like obesity and diabetes the local notions of which are might be quite different from the Western diagnosis (Albuquerque *et al.,* 2014). Computational strategy, a handy tool for drug discovery to pharmaceutical companies, is a promising though underutilised method in the screening of bioactive botanical agents (Arabi and Sardari, 2010). Some studies have previously reported correlation between dietary, epidemiological pattern (Bello *et al.,* 2005) and certain physical attributes like bitter taste (Chen *et al.,* 2015) on one

hand and the presence of targeted pharmacological activities on the other; suggesting that these may serve as bases for developing more robust structured selection method.

In the present study, we explored the use of certain phenotypes (e.g. height, shape of leaf) for screening plants for antiobesity activity, using computer modelling. The modelling is based on a hypothesis that certain physical phenotypes of a plant tend to correlate with certain chemical phenotypes (e.g. presence of a particular phytochemical constituent) if both are products of a common gene (Nachtomy *et al.,* 2007). To the best of our knowledge, this study is the first to explore the possibility of exploring the potential antiobesity activity of botanical agents using phenotypic characteristics as a screening tool.

Materials and Methods

Development of Local Database of Desired Plants

A literature search was performed using Google Scholar and Scirus search engines for medicinal plants that possess antiobesity activities. The search terms used were ("antiobesity" OR "weight lowering") AND "extract". The predefined criteria were to include studies that have been evaluated for antiobesity pharmacological activity with either negative (not active) or positive (active) conclusions at P less than or equal to 0.05 and which include the botanical name of the plant. An excel spreadsheet was used as the database. Information from retrieved studies that were extracted included botanical, common and/or English names of the plant, presence or absence of antiobesity activity, part(s) of the plant used and extraction method (solvent). Furthermore, for all included plants, information about shape of leaf (simple or compound) and height of the plant were obtained from United States Department of Agriculture (USDA, Washinghton DC, www.usda.gv) and 'Plants For A Future' (PFAF, Devon,UK, www.pfaf.org) websites as well as using the above-mentioned internet search engines. These 2 parameters were chosen for modelling because they were considered to be more amenable to objective and consistent intra and interpersonal determination.

Analysis of the Local plant Database for signature characteristics and Plant selection

All the plants in the local database were connoted into forced binomial options of either active or inactive against obesity without consideration for relative potencies because such consideration requires a prior standardisation across studies. Any plant with missing data about the antiobesity activity (due to unavailability) was assumed to be inactive against the pharmacological activity. Although such an assumption may serve as a source of type 1 error (that is, plants that are in fact active may be labelled inactive just because data on them were not available), this was considered acceptable because the overall intention of the model was to be able to select ONLY plants that are active against obesity from any cultivar and not to select ALL plants that are active against obesity in that cultivar. Furthermore, any inaccuracy introduced by the assumption due to an apparent reduction in sensitivity (true positive rate) will be counterbalanced by an apparent proportionate increase in specificity (true negative rate). Any plant with a missing data on the shape of leaf, height of plant, plant part or plant extract, was excluded from any computation involving that parameter.

Drugs and Chemicals

Metformin (Glucophage, Merck) and orlistat (Xenical, Roche Ltd, Switzerland), were procured from a reputable pharmaceutical store. Other chemicals used were of analytical grade.

Criteria for Assessing Parameters of Our Model for Selecting Plants with Activity against Components of Metabolic Syndrome

Receiver Operating Characteristic (ROC) was used to assess the ability of plant height (continuous data) to predict antiobesity activity. High specificity (of at least 90%) was used as one criterion in selecting the values for height to be used for analysis. This is based on the assumption that the cost of commission (i.e. false positive rate) is greater than the cost of omission (false negative rate) and, also, it is well known that increased specificity of any tool used in performing a test tends to decrease the rate of commission (1-specificity). Based on this criterion, only the top 10% of the ROC plot on its extreme left side (with specificity values of 90-100%) was used for analysis, while the middle and the right side of the ROC curve (that fall within specificity of less than 90%) were considered valueless.

For the plant height, the performance of 2 ROC curves (one involving heights which are either less than or greater than various cut off values of height) were compared and the ROC curve with larger partial area under the curve (the top 10% of the plot) was chosen. Youden's index {maximum(sensitivity +specificity-1)} was finally used to select optimum cut off value of height (in m).

For categorical data (i.e. shape of leaf), degree of robustness was used in assessing the option. Robustness analysis, which assesses stability of performance across different scenarios, has recently been advocated as valuable in multicriteria decision analysis (Roy, 1998; Vincke, 1999). It is assumed that the larger the value, the more robust the option is in predicting the antiobesity activity. The shape of leaf with higher total value of {(sensitivity+ specificity)-1} was chosen as the most robust option. For other parameters (part of plant and type of extract), the option with highest frequency of use in % was pre-specified as the choice.

Identification, Extraction and Phytochemical Analysis of the Selected Plants

Five plants with simple leaf and with height shorter than 0.30 m (selected based on the findings of the stochastic model) and five randomly selected plants (serving as control) were used in the study. The plants selected for the study were identified by Umar Muhammad Kebbe and Prof. A.A. Aliero of Botany Department and the Voucher specimen left at the herbarium.

The part of plant and the type of extract with the highest frequency of use among the plants with antiobesity activity were the leaves and the aqueous extract respectively. Accordingly, the leaves of each of the 10 plants used (5 from the stochastic model and five from the control model) were air-dried till constant weight and then reduced to a fine powder (using a pestle and a mortar) followed by aqueous extraction (forty gram of the dried leaves in 200 ml of water) employing plain bottled water. This is followed by a frequent stirring for 30 minutes and subsequent rapid filtration through a clean cloth.

The marc was squeezed to get a combined filtrate, which was stored in a freezer, serving as the stock concentration, for further dilution to required concentration when needed. In order to get an estimate of the stock concentration, ten ml of the combined filtrate was evaporated to a solid mass and weighed. Qualitative phytochemical analysis (El-Olemy *et al.,* 1994) was performed on aqueous extracts of the selected plants for alkaloids, anthraquinones, cardiac glycosides, flavonoids, steroids/terpenoids, saponins and tannins.

Animals

Ninety eight male Wistar rats weighing 135-195g were utilised for the study. First, before starting the study, ethical permission was secured from a departmental committee, Department of Pharmacology and Therapeutics, College of Health Sciences, Usmanu

Danfodiyo University Sokoto Nigeria. The animals were acclimatized for 1 week on high fat diet with access to water ad libilitum.

In Vivo Antiobesity Bioassay

The anti-obesity study was conducted using high-fat diet on all the 10 plants (single dose-incorporated into feeds at 500mg/kg, 7 animals per dose).

Formulation of high-fat diet

High fat diet was formulated by adapting the method used by Yang *et al.,* (2007). Thirty six gram (36 g) of standard chow, 40 g of sheep tallow (animal fat around the loins and kidneys), 4 g of sugar and 20 g of whole milk were mixed, giving 100 g of high-fat diet. Based on the recorded baseline average daily feed intake of the rats (100 g of feed/kg bwt of rats), 500 mg of each of the 10 selected aqueous leaf extracts were incorporated into the high-fat diet. To prevent putrefaction, the feeds were stored in a deep freezer until time for usage.

Design of the experiment for *In Vivo* Antiobesity Bioassay

Ninety-eight (98) male Wistar rats were weighed and then assigned to 14 groups (consisting of 7 animals each) using computer randomisation (employing STATS 2.0). Two groups served as negative controls (to be given standard feed and high-fat feed respectively).

Another 2 groups served as positive controls (given metformin at 0.20 g% and orlistat at 0.04 g% respectively); and the remaining ten groups function as treatment groups (to be given one extract each).

The animals were first fed ad libitum on high fat diet for 1 week, during which they were determined to, on the average, ingest 100g of feed per kg bwt per day. This is similar to the finding of Calapai *et al.,* (1999). Thereafter, the fourteen groups of animals were treated as follows: 2 negative controls (fed either a standard formula diet {Grand cereals, Jos} or high fat diet, 2 positive controls (treated with orlistat 400mg per kg of high fat feed ({equivalent to 40 mg per kg bw of rat}) and metformin 2000 mg per kg of high fat feed {equivalent to 200 mg per kg bw of rat}) and 10 treatment groups that were given 500mg of each of the 10 extracts per 100 g of high fat diet ({equivalent to 500mg per kg bw of rat}).

Because this stage of the study was exploratory, single dose per plant was considered more cost effective as this will reduce the cost of attrition. Furthermore, it matches the single dose treatment given to controls. Also, 500 mg/kg was chosen as the single-dose screen because most efficacious extracts almost always show activity at this dose.

Animals were weighed weekly and their daily food intake were also carefully assessed and documented. After 7 weeks treatment, the animals were anaesthesized with chloroform vapour in a gas jar and then sacrificed.

The weights of white adipose tissue (perirenal and epididymal) were measured (in g /100 body weight) after dissection.

Statistical Analysis

The experimental data was expressed as mean ± SD. Results were analysed using GraphPad Prism software version 6. Comparison of the different group means was performed using either Student t test (for 2 groups) or ANOVA followed by Tukey-Kramer post hoc test (for more than two groups), with $P \leq 0.05$ considered to be significant.

Results and Discussion

Development of Local Database of Desired Plants

A total of 495 plants were retrieved, of which 48 were reported to be active against obesity, while 447 plants were not documented to possess antiobesity activity. Of the 495 plants retrieved, the average heights and shape of leaf of 404 (91 missing) and 424 (71 missing) respectively were obtained.

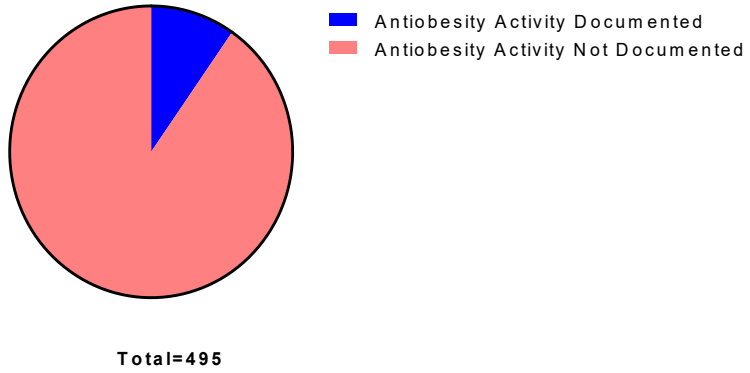
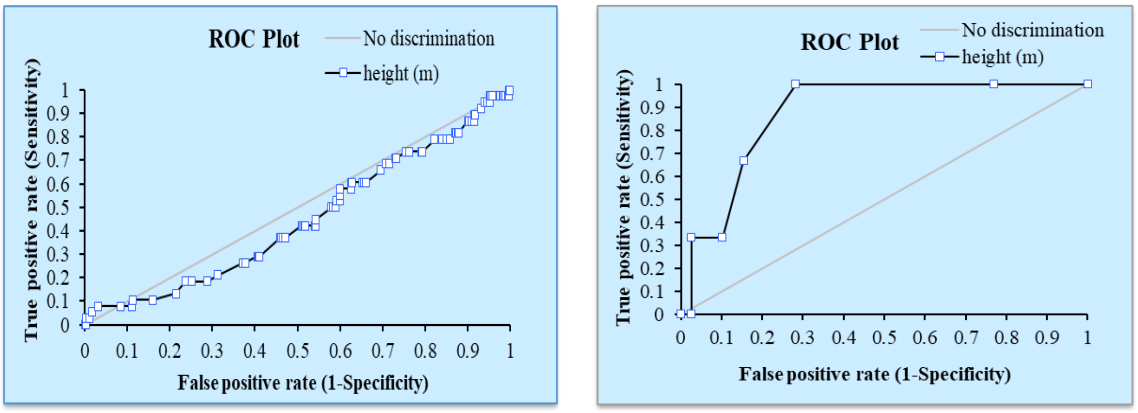


Figure 1. A pie chart showing the number of plants documented to possess antiobesity activity out of the 495 plants retrieved

Plant Height and shape of leaf as predictors of Antiobesity Activity

Considering only the top 10% of the whole ROC plot (specificity ≥90%; Fig. 2A), short plants (<0.40 m) performed significantly better than random selection (pAUC of 0.88 ± 0.068; 95% CI= 0.74-1.00; P<0.0001) in predicting antiobesity activity (Fig. 2B). In contrast, the performance of tall plants (>15.00 m) did not differ from random (pAUC of 0.42 ± 0.125; 95% CI= 0.17-0.66; P=0.7423; Fig. 3B) when the top 10% of the whole ROC plot was considered. The Youden's index, 0.048, was obtained at a plant height <0.3m (Table 1). Concerning the shape of leaf, simple leaf performed better in predicting the antiobesity activity of plants (Youndens index: 0.100; LR(+): 1.14) than compound leaf (Youndens index: -0.100; LR(+): 0.63) (Table 1).

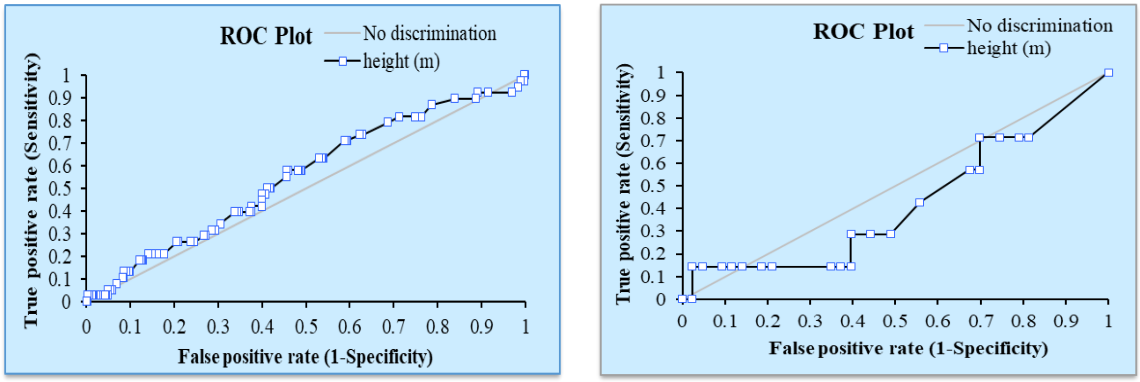


A **B**

Figure 2. The Receiver Operating characteristic (ROC) curve for the anti-obesity activity of plants with a height shorter than various cut off values.

(A) The whole ROC plot.

(B) Partial ROC plot showing the top 10% of the whole plot (cut off height < 0.40 m).



A **B**

Figure 3. The Receiver Operating characteristic (ROC) curve for the anti-obesity activity of plants with a height taller than various cut off values.
(A) The whole ROC plot.
(B) Partial ROC plot showing the top 10% of the whole plot (cut off height > 15.0 m).

Table 1. Sensitivity, specificity, and LR (+) of using the height of plants or either of the 2 options for the shape of plant leaf as a selection tool for antiobesity activity

Tool for Plant Selection			Sensitivity	Specificity	Sensitivity + Specificity-1	Likelihood Ratio (+)
Height of plants	Short plants (positive <cut off) within the range of heights with acceptable specificity (90-100%)	<0.30 m	0.079	0.969	0.048	2.55
	Tall plants (positive > cut off) within the range of heights with acceptable specificity (90-100%).	>18.00 m	0.132	0.915	0.047	1.56
Shape of leaf	Use of Simple leaf		0.833	0.267	0.100	1.14
	Use of Compound leaf		0.167	0.733	-0.100	0.63

Part of plant and solvent for extraction as Predictors of activity against components of the metabolic syndrome

Using 5% (i.e. five per cent of the total parts of plants used) as the critical limit for inclusion, the following were the 5 most commonly used parts of plants among the plants with antiobesity activity, in descending order of frequency: leaf (23.6%) > root (18.2%) > seed (10.9%) > fruit (7.3%) > rhizomes (5.5%).

For the methods of extraction, the order of frequency was aqueous (43.3%) > ethanolic (26.7%) > hexane (6.7%).

Plants Selected for the study

A total of twenty three plants were selected. Fifteen (15) plants were chosen using the stochastic model (simple leaf and a height ≤0.30 m), while 8 plants were selected randomly. Subsequent eliminations (Figure 4) yielded 10 plants with 5 plants allocated to the model, while the remaining 5 plants serve as control.

The identity of the plants are listed in Table 1

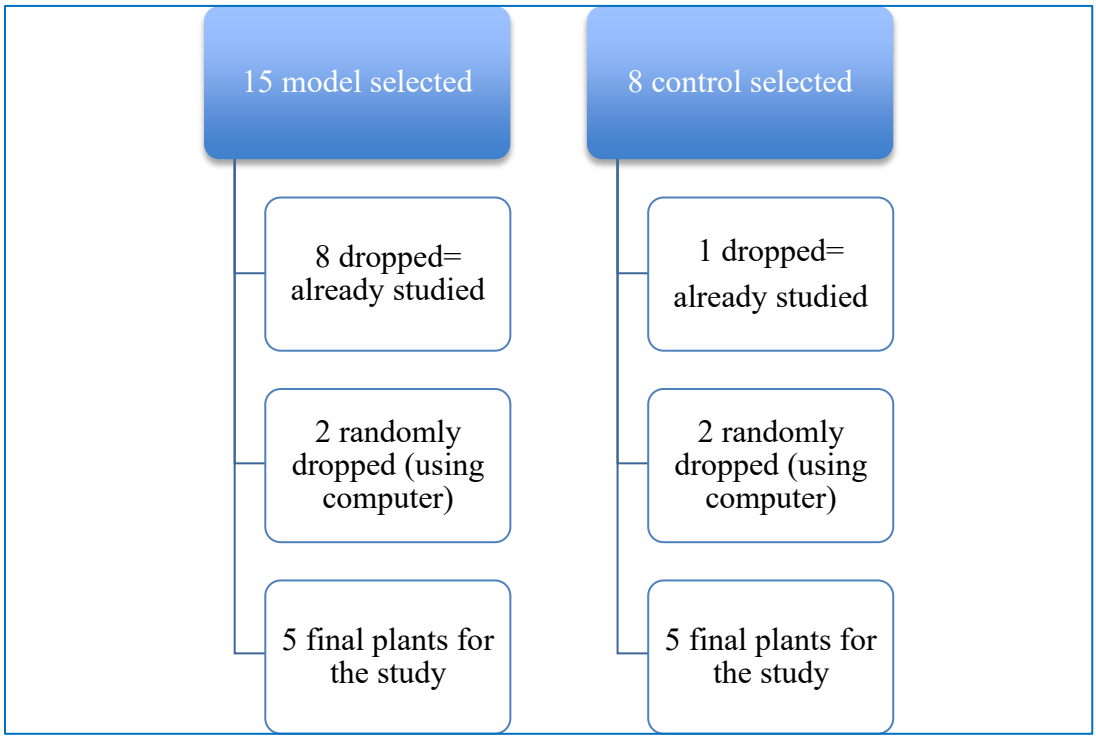


Figure 4. Plants selected versus those subsequently eliminated early

Phytochemical Analysis of Aqueous Extracts of the Selected Plants

The phytochemical analysis of the 10 plants selected are summarised in Table 2.

Table 2. Phytochemical constituents of selected plants

Plant species/Vernacular name/ Voucher no.	Result of qualitative Phytochemical Analysis						
	Tannins	Alkaloids	Saponins	Flavonoids	Steroids/ Terpenoids	Anthra-quinones	Glycosides
Selected based on stochastic model							
Commelina diffusa/ Balaasaya/034	Negative	Negative	Positive	Positive	Positive	Negative	Positive
Vigna subterranea/ Kwaaruru/041	Positive	Positive	Positive	Positive	Negative	Negative	Positive
Azolla Africana/ Kainuwa/042	Negative	Negative	Positive	Positive	Negative	Negative	Negative
Byrsocarpus coccineus/ Tsaamiyar kasa/036	Positive	Positive	Positive	Positive	Positive	Negative	Negative
Phyllanthus Pentandrus/ Geron tsuntsaye/ V/N/PCG/UDUS/EUP/ 0010	Positive	Negative	Positive	Positive	Positive	Negative	Positive
Selected randomly							
Solanum anomalum/ Gautan kaji/ 045	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Aeschynomene indica/ Zamarke/ 052	Positive	Negative	Positive	Positive	Positive	Negative	Positive
Leptadenia hastata/	Positive	Positive	Negative	Positive	Positive	Negative	Negative

Yadiya/ V/N/PCG/UDUS/ASC/ 0002							
Bauhinia rufescens/ Jirga/ 053	Positive	Positive	Positive	Positive	Positive	Negative	Positive
Cassia italica/ Fatakko/035	Positive	Negative	Positive	Positive	Negative	Negative	Negative

Effect of the Aqueous Leaf Extracts of the Selected Plants on Body Weight Gain, Food Intake and White Adipose Tissue Mass

There was no significant difference in body weight between the groups at baseline (Table 2). By the end of the experiment, high fat diet (HFD)- fed rats exhibited a significant increase in body weight gain and white adipose tissue mass. Administration of high-fat diet (which can be formulated by mixing standard chow with added fat) is a common method of inducing obesity in rodents. Sheep tallow (derived from perirenal fat by low-temperature cooking), consists, predominantly, of saturated fat, which is more likely to induce weight gain and insulin resistance in rodents than unsaturated fat (Buettner *et al.,* 2006).

The choice of the level of fat to be added to the diet requires a balanced decision. On the one hand, adding small amount of fat is likely to result in failure to induce obesity, while on the other hand adding too much fat may, unintentionally, render the animals deficient in nutrients particularly protein (Gajda *et al.,* 2007).

The weight gain by animals is dependant on the level of fat contained in the diets used in obesity research, which ranges between 30-50% (high-fat diet) to more than 50% (very high-fat diet) (Gao *et al.,* 2002). In order to strike such a balance between optimising the chance of inducing obesity as well as minimising the attendant risk described above, a moderate amount of fat (about 40%) has been selected in the model used in this study.

The standard chow used contains 7% fat and 15% protein. By adding moderate amount of fat (40g per 100g diet and whole milk powder (20 g per 100g diet) the resultant high fat diet contains about 11% protein (since the whole milk contains 27% protein), a level, though deficient, that may not lead to deleterious effects (Balakrishnan *et al.,* 1985; Matsuda *et al.,* 2004).

The body weight gain was significantly ($P \leq 0.05$) reduced after 7 weeks treatment with each of the five extracts from plants selected by stochastic model but only three of the extracts from plants selected randomly. The effect was significantly ($P \leq 0.05$) greater than that of orlistat (which showed no significant difference from that of HFD control) and tend (though not significantly) to be greater than of metformin (which was significantly lower than that of HFD control).

As a group, animals administered with plants selected by stochastic model showed significantly less weight gain than animals administered with plants selected by the control model (Figure 5).

Some of the phytochemicals identified in the extracts have been reported to exhibit antiobesity properties (Schwartz and Lewbart, 1997; Hu *et al.*, 2012; Hossain *et al.,* 2016) and any of them, individually or in combination might be responsible for the observed antiobesity activity. The failure of orlistat to reduce body weight gain in HFD-fed rats as observed in this study agrees with the finding of Maahs *et al.,* (2006) showing that orlistat

failed to reduce BMI in obese adolescents. The drug acts by inhibiting pancreatic and gastric lipases, resulting in a decrease in the absorption of dietary lipids (Nightingale, 1999).

Also, the above-mentioned extracts resulted in a significant (P≤0.05) reduction in food intake, greater than that of orlistat and metformin (which exhibited effect not significantly different from HFD control). However, only one extract each from the plants selected based on the stochastic and control models showed significant reduction in white adipose tissue mass with similar efficacy to that of the standard drug orlistat, which was also effective (Table 3).

The food intake-reducing effect of the extracts as observed in the current study suggests that reduction in food consumption, might at least be partly contributory to the body weight-reducing effect of the extracts.

Table 3. Effects on body weight gain, food intake and white adipose tissue weight of the 10 selected plant extracts

Treatment groups	Baseline Body weight (g)	Body Weight gain at week 7 (g)	Food intake (gram /animal/day)	White adipose tissue weight (g/100 g body weight)
NC	148.65±16.50	30.9± 8.64 ^{bc}	16.3± 1.7 ^{ab}	1.1± 0.3 ^{c}
HFD	166.13±21.58	70.5± 13.1 ^{a}	17.9± 1.9 ^{a}	5.0± 1.2 ^{a}
ORL+ HFD	160.04±21.53	53.50± 9.83 ^{ab}	19.0±2.1 ^{a}	2.1± 0.6 ^{bc}
MTF+ HFD	175.11±20.51	36.0± 17.1 ^{bc}	18.7± 1.8 ^{a}	3.4±1.00 ^{ab}
COM+ HFD	161.56±23.04	16.03± 16.67 ^{c}	12.8± 1.6 ^{bc}	4.10± 2.00 ^{a}
VIG+ HFD	170.63±27.54	26.26± 12.03 ^{c}	12.1± 1.8 ^{bc}	4.1±1.0 ^{a}
AZO+ HFD	170.16±23.47	22.87± 10.32 ^{c}	14.0± 1.0 ^{b}	4.2±0.8 ^{a}
BYR+ HFD	158.00±20.82	28.13± 14.93 ^{c}	11.6± 1.5 ^{bc}	3.5±0.4 ^{a}
PP+ HFD	166.96±21.48	17.88± 10.40 ^{c}	10.0± 1.2 ^{c}	3.1±0.8 ^{b}
SOL+ HFD	170.05±19.65	52.0± 14.6 ^{ab}	18.4± 2.0 ^{a}	3.5±1.5 ^{ab}
AES+ HFD	157.74±9.65	26.2± 12.2 ^{c}	11.4± 1.3 ^{bc}	3.4±1.0 ^{ab}
LEP+ HFD	147.01±10.16	18.4± 9.9 ^{c}	11.8± 1.4 ^{bc}	2.6±0.6 ^{bc}
BAU+ HFD	167.79±22.52	26.7± 13.6 ^{c}	14.3± 1.6 ^{b}	3.5±0.8 ^{ab}
CAS+ HFD	170.50±12.05	76.6± 15.1 ^{a}	18.1± 2.2 ^{a}	4.3±1.2 ^{a}

Values represent the means ± standard deviation (n = 7). Mean values with a common lower case superscript letters in the same column are not significantly different at p≤0.05.

Key: AES: *Aeschynomene indica*; AZO: *Azolla Africana*; BAU: *Bauhinia rufescens*; BYR: *Byrsocarpus coccineus*; CAS: COM: *Commelina diffusa*; HFD: high fat diet; LEP: *Leptadenia hastata*; MTF: NC: Normal control; ORL: orlistat; PP: *Phyllanthus pentandrus*; SOL: *Solanum anomalum*; VIG: *Vigna subterranean*

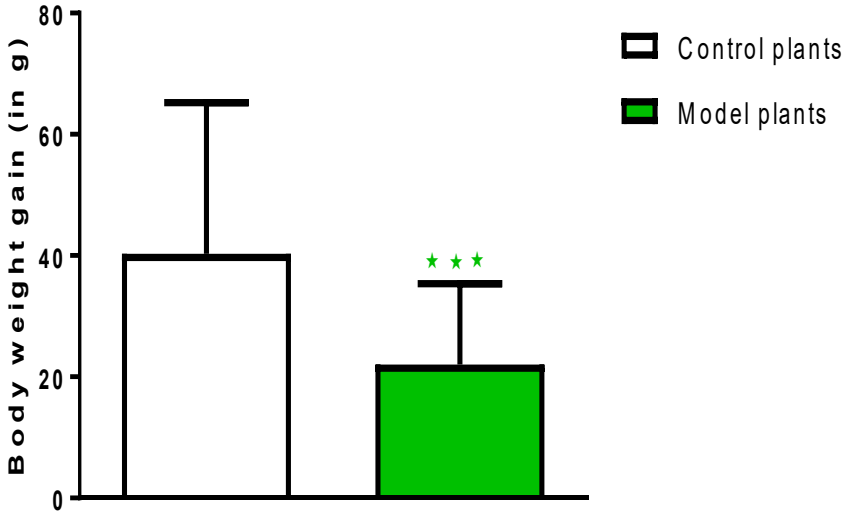


Figure 5. Comparison of body weight gain between animals dosed with extracts of plants selected randomly or using the stochastic model. Values represent the means ± standard deviation using Student t test (n= 35 rats each). * signify P<0.001.**

To the best of our knowledge, this is the first time stochastic modelling was applied to prediction of pharmacological activity of plants.

The present study is also the first to report the antiobesity activity of any member species of the following genera--*Aeschynomene*, *Leptadenia, Azolla* and *Byrsocarpus.* Also, although some members of the genus *Vigna* (Liu *et al.,* 2017), *Phyllanthus* (Ahmed, 2017), *Bauhinia* (El-Wakf *et al.,* 2019) and *Commelina* (Nagai *et al.,* 2016) have been previously documented to possess antiobesity activity, the current study is the first to report the antiobesity activity of the following species--*Vigna subtarranea*, *Phyllanthus pentandrus*, *Bauhinia rufescens* and *Commelina diffusa*.

Our study has some limitations. Firstly, the phytoconstituents in the extracts have not been characterised using using high-performance liquid chromatography and mass spectrometry. The authors have, however, identified certain phytochemicals that have been previously documented to exhibit antiobesity properties.

Secondly, the authors only investigated the effect of a single dose (500 mg/kg/day) of each of the 10 plant extracts (chosen based on a preliminary study showing the extracts to be well tolerated by the rats at that dose).

Conclusion

Stochastic modelling using phenotypic characteristics was found to predict antiobesity activity in plant extracts; thus, it may be potentially useful in discovering plants effective in treating various disease conditions.

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Conflicts of interest
The authors declare no conflicts of interest.

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