*Research Article*

# Formulation of category-2 hypoglycemic Improved Traditional Medicines from selected Cameroonian medicinal plants: *Mangifera indica*, *Persea americana* and *Ageratum conyzoides*

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## Abstract

Diabetes mellitus is the fourth leading cause of death worldwide, and constitutes a major public health crisis. Management of the adverse condition relies mostly on synthetic drugs such as metformin, glibenclamide and insulin. However, treatment with synthetic drugs is challenged by side effects and high cost especially to patients in low-income countries like Cameroon. This study set out to formulate Improved Traditional Medicines (ITMs) in the form of capsules from lyophilized aqueous leaf extracts of *Ageratum conyzoides*, *Mangifera indica* and *Persea americana*, in combination, coded as ITM-1, and leaf extract of *Mangifera indica* alone, referred to as ITM-2. Phytochemical profiling of individual extracts was carried out using standard methods, while their antioxidant activity was evaluated *in vitro* by the 2,2-diphenyl-1-picryhadrazyl reducing (DPPH) assay and the Ferric (Iron) reducing antioxidant power assay (FRAP). Capsule formulation was guided by findings from prior Physicochemical and pharmacotechnical analysis of individual extracts and their combination. Safety studies were carried out both *in vitro* (cytotoxicity testing in Vero cells); and *in vivo* (acute toxicity tests using the mouse model). Preliminary evaluation of the antidiabetic potential of the formulated ITMs was achieved through determination of their acute hypoglycemic and antihyperglycemic properties in Wistar rats (Oral Glucose Tolerance Test). Major classes of phytochemicals detected were alkaloids, phenols, tannins, flavonoids, anthocyanins, tri-terpenes and anthraquinones. The DPPH and FRAP assays showed dose-dependent antioxidant activity for ITM-1 and ITM-2. Both ITMs showed no toxic effects, be it *in vitro,* (Cytotoxic Concentration 50% - $CC_{50}$>1000 µg/mL in cells) or *in vivo.* At the dose of 25 mg/Kg, both ITMs exhibited significant hypoglycemic effects in Wistar rats. The ITM-2 capsules completely suppressed post-prandial glucose peak in rats as compared to the negative control (distilled water), ITM-1 capsule was able to cause restoration of the glucose levels to normal levels after 120 minutes.

**Key words:** Diabetes Mellitus, Antidiabetic activity, Improved traditional medicines medicinal plants, *Ageratum conyzoides*, *Mangifera indica*, *Persea americana.*

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**RESUME**

Le diabète sucré est la quatrième cause de mortalité dans le monde et constitue une crise majeure de santé publique. La gestion de cette maladie repose principalement sur des médicaments synthétiques tels que la metformine, le glibenclamide et l'insuline. Cependant, le traitement avec des médicaments synthétiques est confronté à des effets secondaires et à un coût élevé, en particulier pour les patients des pays à faible revenu comme le Cameroun. Cette étude a pour but de formuler des médicaments traditionnels améliorés (MTA) sous forme de gélules à partir d'extraits aqueux lyophilisés de feuilles *d'Ageratum conyzoides*, de *Mangifera indica* et de *Persea americana*, en combinaison, codés sous le nom de MTA-1, et d'extraits de feuilles de *Mangifera indica* seuls, appelés MTA-2. Le profilage phytochimique des extraits individuels a été réalisé à l'aide de méthodes standard, tandis que leur activité antioxydante a été évaluée *in vitro* par le test de réduction du 2,2-diphényl-1-picryhadrazyle (DPPH) et le test du pouvoir antioxydant réducteur du fer (FRAP). La formulation des gélules a été guidée par les résultats de l'analyse physicochimique et pharmaco-technique des extraits individuels et de leur combinaison. Des études d'innocuité ont été réalisées à la fois *in vitro* (tests de cytotoxicité sur cellules Vero) et *in vivo* (modèle de toxicité aiguë chez la souris). L'évaluation préliminaire du potentiel antidiabétique des MTI formulés a été réalisée par la détermination de leurs propriétés hypoglycémiques et anti-hyperglycémiques aiguës chez des rats Wistar (test de tolérance au glucose par voie orale). Les principales classes de substances phytochimiques détectées étaient les alcaloïdes, les phénols, les tanins, les flavonoïdes, les anthocyanes, les triterpènes et les anthraquinones. Les tests DPPH et FRAP ont montré une activité antioxydante dose-dépendante pour ITM-1 et ITM-2. Les deux MTI n'ont montré aucun effet toxique, que ce soit in vitro (concentration cytotoxique 50 % - CC50 >1000 µg/mL dans les cellules) ou i*n vivo*. A la dose de 25 mg/Kg, les deux MTI ont montré des effets hypoglycémiques significatifs chez les rats Wistar. Les capsules ITM-2 ont complètement supprimé le pic de glucose post-prandial chez les rats par rapport au contrôle négatif (eau distillée), tandis que la combinaison de capsules ITM-1 a été en mesure de restaurer les niveaux de glucose à des niveaux normaux après 120 minutes.

**Mots clés :** Diabète sucré, activité antidiabétique, médicaments traditionnels améliorés, *Ageratum conyzoides*, *Mangifera indica*, *Persea americana*.

## INTRODUCTION

Diabetes mellitus (DM) is a global public health challenge which stands as a serious economically devastating problem, holding high epidemic proportions in both affluent and developing countries. It is a chronic metabolic disorder characterized by hyperglycemia which subsequently leads to complications in the heart blood vessels, eyes, kidneys and nerves (WHO, 2022).

There are about 422 million cases of DM globally with a mortality rate of 1.5 million annually (WHO, 2022). As a very common chronic disease, diabetes is becoming the fouth 'killer' of mankind, after cancer, cardiovascular and cerebrovascular diseases, because of its high prevalence, morbidity and mortality (IDF, 2021). Two major types of diabetes are type 1 and type 2. Type 1 diabetes results from inability of the pancreas to secrete sufficient insulin for regulation of glucose uptake. As such, post prandial glucose production levels remain consistently elevated while glucose uptake by peripheral cells does not increase. Type 2 diabetes stems from insulin resistance by liver and muscle tissue (Defrenzo, 1981; Bonadonna *et al.*, 1996). This insulin resistance is influenced by defects in insulin receptor function, defects in insulin receptor signal transduction pathway, defects in glucose transport and phosphorylation, and defects in glycogen and

glucose oxidation (Khalid *et al.,* 2021; Petersen and Shulman, 2018). For both type 1 and type 2 diabetes, the outcome is excessive endogenous glucose production and the absence of glucose uptake which contribute equally to the body's inability to use glucose as a primary source of energy (Bhavna *et al.*, 2008). Hyperglycemia results in complications of diabetes such as macro vascular complications (neuropathy, and nephropathy) and microvascular complications (retinopathy, cardiovascular diseases, diabetic ulcers and subsequent amputation).

So far, type 1 diabetes is mostly treated by insulin therapy, while for type 2, a wider range of chemotherapies exist (Defronzo, 1999). However, both insulin-therapy and other existing drugs are seriously limited by side effects which can range from simple diarrhea, abdominal discomfort, obesity, to anemia, pulmonary edema and hypoglycemic coma (Cheng and Fantus, 2005; Bhavna *et al.*, 2008). Moreover, there is as yet no effective curative drug for diabetes mellitus.

Due to the increasing incidence of diabetes in Cameroon, coupled with limited financial power to afford existing drugs, especially in rural settings, there has been a massive push towards the use of herbal medicines for the treatment of diabetes in recent decades (Tsabang *et al.,* 2017). The formulation of polyherbal teas is a traditional therapeutic strategy which exploits the combination of several medicinal herbs to achieve extra therapeutic effectiveness, usually known as polypharmacy or polyherbalism. Several studies have revealed that plants of varying potency when combined may theoretically produce a better result, as compared to individual use of the plant (Subramani *et al.*, 2014).

Recent studies assessed the antidiabetic activity and safety of a mono-herbal tea prepared from the leaves of *Mangifera indica* and a polyherbal tea prepared from a combination of the leaves of *Ageratum conyzoides*, *Persea americana* and *Mangifera indica* on the type 2 diabetes Wistar rat model (Zofou *et al.*, 2023). The results obtained revealed that the mono-herbal tea of *M. indica* leaf at 20mg/Kg showed acute hypoglycemic and postprandial peak suppression effects, while the polyherbal tea of *M. indica*, *P. americana* and *A. conyzoides*, at 25mg/Kg, was able to return the glucose levels to normal 30mins after the glucose peak. Both traditional preparations also showed significant efficacy in type 2 DM rat model, with no significant acute toxicity (Zofou *et al.*, 2023). In addition, significant hepato-protective and nephroprotective effects of these teas were recorded in diabetic rats. Despite these promising findings, the use of teas in the treatment of diabetes is marred by setbacks such as short shelf life, bulkiness, and lack of standardized preparation procedures by patients. The aim of this work was to develop and test category 2 Improved Traditional Medicines (ITM) from these two traditional preparations using the reverse pharmacology approach.

## MATERIAL AND METHODS

### Harvesting and processing of plant materials

Leaves of *A. conyzoides*, *M. indica*, and *P. americana* were harvested between 5am and 7am local time in Buea, South West Region of Cameroon during the month of December (dry season). Sample plant materials were used for identification at the National Herbarium, Yaounde. This was done by comparing with voucher specimen previously registered under Collector/sample numbers SCA No.353/No32875HNC, MPOM Benoit No448/No 19050SRF/Cam, SCA No235/33945HNC for *M. indica*, *A. conyzoides* and *P. americana*, respectively. The leaves were washed, weighed and 2000 g air dried for 3 weeks and later ground to powder using a high-speed blender. Water decoctions were prepared based on the local traditional practice in communities in the region, as described by Zofou *et al.,* 2023 whereby, two teaspoons of powder (equivalent to 10g for the herbal tea of the crushed leaves) is generally

infused in one teacup of water (200 mL), for about 5 mins. The prepared decoctions were then filtered using Whatman paper No.1, and lyophilized to powder.

**Phytochemical characterization of freeze-dried extracts**

The extracts were screened for detection of different chemical families according to the methods previous described by Odebiyiand Safowara (1978). In brief, phenolic compounds were detected using the ferrocyanide reaction; triterpenes and sterols were revealed by their reactivity with anhydrous acetate and sulphuric acid. Alkaloids were detected using Mayer reagent, whereas the presence of saponins was revealed based on their foaming property. Tannins and flavonoids were revealed using ferric chloride and hydrochloric acid, respectively. Anthraquinones were detected in extracts by the chloroform/petroleum system, while the presence of lipids was assessed on filter paper.

Quantitative analyses of tannin, saponin, alkaloid and phenolic compounds were carried out as previously described by Teugwa *et al.* (2013), with slight modifications.

***In vitro* antioxidant activity**

The *in vitro* antioxidant activity of the lyophilized extracts were determined using the DPPH (2,2-diphenyl-1-picryhadrazyl) assay and the Iron reducing antioxidant power assay.

**DPPH (2,2-diphenyl-1-picryhadrazyl assay):**The free radical scavenging activity of all lyophilized extracts was determined using the method described by Katalinie *et al.* (2004) with some modifications. Summarily, a volume of 50 µL of different concentrations (2, 4, 8, and 16 g/L) of each extract was added to 1.950 mL of the freshly prepared ethanoic solution of DPPH. The mixture was incubated in the dark for 30 minutes, then the absorbance was read at 515 nm against the blank consisting of DPPH and different concentrations of vitamin C (5-50 µg/mL).The results were expressed as percentage inhibition of the DPPH radical according to the following formula:

$$\%\ inhibition\ of\ DPPH\ radical = \frac{[Ac(0) - Ae(t)] * 100}{Ac(0)}$$

**Ac (0)** = Control absorbance at t = 0 min.

**Ae (t)** = Assay absorbance after 30 min incubation.

**The ferric reducing antioxidant power (FRAP) assay:** This test assessed the potential of antioxidants to reduce ferric iron ($Fe^{3+}$) to ferrous iron ($Fe^{2+}$). In the presence of antioxidants, potassium ferrocyanide, trichloroacetic acid, and iron chloride form a blue complex absorbing at a wavelength of 700 nm where increase in absorbance is proportional to the reducing power of the sample. Briefly, to 1.25 mL of phosphate buffer was added 1.25 mL of potassium ferrocyanide and 0.5 mL of extracts (2-4-8 and 16 mg/mL). This was then incubated at 50°C for 20 min. Subsequently, 2.5 mL of trichloroacetic acid was added and the reaction mixture was centrifuged at 3000 rpm for 10 min. Then 1.25 mL of the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL of ferric chloride. The absorbance of the final solution was read at 700 nm against the blank prepared in parallel by replacing the extract with distilled water (Jayaprakash *et al.*, 2001). The results were expressed as a percentage reduction according to the following formula:

$$\%\ FRAP\ reduction = \frac{[ODtest - ODcontrol]x100}{ODtest}$$

Where ODtest = Optical density of test;
ODcontrol = Optical density of control

**Formulation of capsules**

In order to formulate the improved traditional medicines, two capsule formulations were envisaged, with one containing active extract of *M. indica* leaf alone, and a second one including a

combination (equal amounts) of active leave extracts of *M. indica*, *P. americana* and *A. conyzoides* leaves. The choice of this pharmaceutical form was justified by the fact that capsules are tasteless, relatively easy to swallow, and relative cheap in terms of preparation cost. Overall, the capsule formulation process was carried out as summarized in Figure 1.

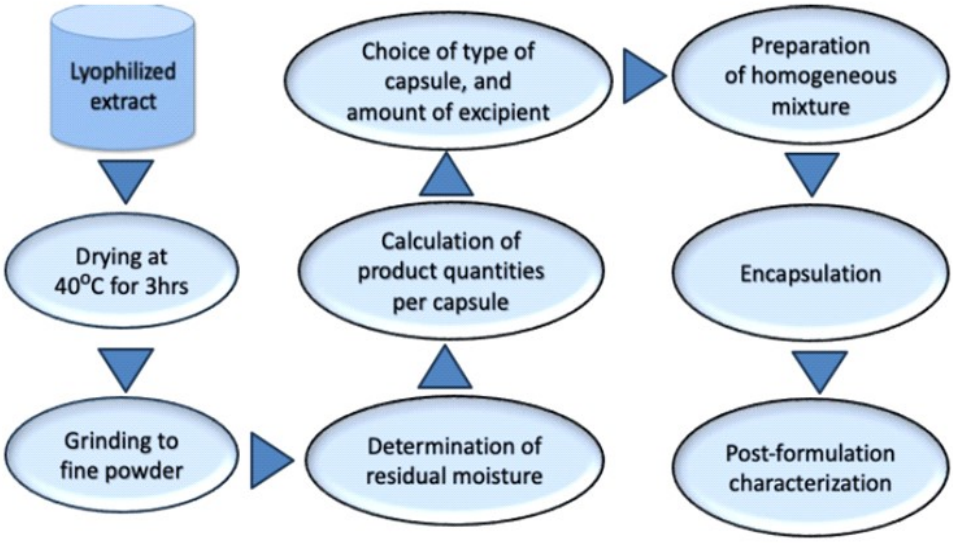


**Figure 1.** Capsule formulation process

The theoretical weight of a capsule was determined from the therapeutic dose of 25mg/Kg per day for an adult, which was informed by mean doses of the monoherbal and polyherbal teas previously evaluated for their antidiabetic activity (Zofou *et al.*, 2023). The lyophilized extracts were dried at 40°C for 3hrs and ground to obtain loose powders, then mixed with corn starch as binding agents till a uniform mass was obtained. Size 00 capsules (Volume 0.9ml, overall length 23.4mm, external diameter 8.56mm) were used, the hard vegetarian capsule shells (size 00) were then filled with the total powder, using a semi-automatic capsule filling machine to obtain the formulation.

**Quality characterization of the formulated capsules**

The quality control tests were performed according to the European Pharmacopoeia 10th edition, these tests checked the capsules for residual moisture content, mass uniformity, pharmacopoeia standards of mass uniformity, and disintegration time.

**Residual moisture content of powders:** The residual moisture content of the ITMs was determined by heating the different formulations under vacuum, followed by moisture content calculation based on the weight loss of the product during the drying cycle. Briefly, the capsules content sample was transferred into a pre-weighed vial "A" and quickly weighed. The weight was recorded in the category "B". The vial and its angled stopper were then placed in a vacuum oven set to < 2.5kPa and 60° ± 3°C. After a waiting period of at least 3hr the vacuum pump was turned off and dry air introduced into the oven until the pressure inside the oven was equal to atmospheric pressure. The vial was then capped while it was still warm and transferred to a desiccator then let cool to room temperature for two hours to achieve a consistent weight. The vial was then weighed again and recorded in category "C". The residual moisture was then determined using the following formula:

$$Residual\ moisture\ (\%) = \frac{[B - C]x100}{B - A}$$

Where A is the weight of the empty container.

B minus A is the weight of the sample before the determination.
B minus C is the weight equivalent to the residual moisture of the sample.

**Weight variation (mass uniformity) test:** The test for uniformity of mass measures the variability in the amount of powder contained in each sample (Mahmud *et al.*, 2013). The average weight of capsules was obtained by randomly selecting 20 capsules and weighing them on an electronic balance. Weight variation was calculated as follows;

$$Weight\ variation = \frac{(Weight\ of\ capsule - Average\ weight\ of\ capsules)x100}{Average\ weight\ of\ capsules}$$

**Interpretation:** The mass uniformity test was considered not compliant if:
(a) Greater than 2 of the 20 capsules show a difference: (i) Greater than 10% of the theoretical mass less than 300mg; (ii) Greater than 7.5% of the theoretical mass is greater than 300mg
Or
(b) If only 1 capsule has a difference: (i) Greater than 20% of the theoretical mass is less than 300mg; (ii) Greater than 15% if the theoretical mass is greater than 300mg.
The test was considered compliant of all the weighed capsules were in the range: (i) Capsules of less than 300mg; difference of 10 without any capsule having a difference of more than 20%, (ii) Capsules of more than 300mg; difference of 7.5 without any capsule having a difference of more than 15%.

**Capsule disintegration time:** The disintegration test was carried out as previously described (Nnanga *et al.*, 2018). Briefly, five capsules at random, were each placed into a tube in the basket-rack assembly and a disc added to each of the tubes. The assembly was then suspended in the beaker containing 0.1M HCl as the immersion fluid, maintained at 37°C. Disintegration was achieved when there was no residue on the grid or only coating fragments remain. Disintegration time was then recorded. According to the standard, the disintegration time for an uncoated tablet is 15 minutes or less.

**Safety evaluation of the ITM capsules**

The assessment of the safety of the formulated capsules was carried out using both *in vitro* cytotoxicity assay and *in vivo* rodent models.

***In vitro* cytotoxicity evaluation with Vero cells:** Viability of the cells was assessed by XTT (2,3-Bis-(2-Methoxy-5-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)-basedcell proliferation assay (X12223) according to the manufacturer 's instructions. Normal African green monkey kidney epithelial (Vero) cells were obtained from American Type Culture Collection (ATCC), and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 20 mM HEPES and 1% penicillin–streptomycin. Confluent cells (about 90% viable) were trypsinized and seeded at a density of 10,000 cells/well (total volume of 100ìL) in 96-well plates and incubated for 24hr prior to drug treatment. Two-fold serial dilutions of the compounds (1000 – 7.8125ìg/mL) were added to the plates and incubated under humidified conditions at 37°C for 48 h. Absorbance of the formed formazan product was measured at 450 and 620 nm wavelengths using a MULTISKAN FC microtiter plate reader. Absorbance values were obtained following background subtraction at 620 nm and percent inhibition of growth computed. Dose–response graphs were plotted using GraphPad Prism v.8 and $CC_{50}$ values obtained.

***In vivo* acute toxicity evaluation:** The acute toxicity of the 2 capsules was assessed using both adult male and female Balb/c mice, according to Organization for Economic Cooperation and Development (OECD-425) guidelines for testing of chemicals (OECD, 2001; Zofou *et al.*, 2017). The animals of each sex were randomly divided

into 3 groups of 5 each, making a total of 6 groups which were kept in their respective cages for 5-days acclimatization. Prior to the experiment, the animals were all fasted overnight for 12h and animals in the test groups received through oral gavage, either the ITM-1 or ITM-2 capsules at a dose of 2000 mg/kg, while the animals in the control group were administered the vehicle (distilled water 10 ml/Kg). The mice were then observed for 4 hours following administration of treatments, and periodically every day for 14 days to detect any physical or behavioral changes. The animal feed was composed of 5Kg corn, 1Kg wheat bran, 1Kg of powdered soya beans, 1Kg of powdered fish, 1Kg of powdered bone, 5g of iodized salt and 2g of vitamin pre-mix. Rats were given food and water *ad libitum*. The mortality, food intake and body weight were monitored. The activeness, loss of hair, change in physical appearance and behavior were also observed during the experiment.

**Antihyperglycemic and hypoglycemic activity of capsules**

Upon formulation of the capsules from lyophilized extracts of *M. indica* and a combination of *A. conyzoides*, *M. indica and P. americana*, their acute antidiabetic activities were assessed using the oral glucose tolerance test (OGTT) as described earlier (Zofou *et al*., 2017). A total of 20 rats of 10 weeks old with comparable body weights between 150-200 g were used for this experiment, and they were distributed into 4 groups of 5 rats each as illustrated in table 1:

**Table 1:** Treatment in different experimental OGTT groups

| Experimental group | Treatment |
|---|---|
| Group 1 (Negative control) | distilled water (5mL/Kg) |
| Group 2 (Positive control) | Glibenclamide (10 mg/kg) |
| Group 3 (ITM-2): | ITM-2(25 mg/kg):Capsules of *M. indica*lyophilized leaf water extract |
| Group 4 (ITM-1) | ITM-1 (25 mg/kg): Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts |

The rats were fasted overnight prior to commencement of the test. From each animal's tail, a drop of blood sample was collected and applied on the strip of a glucometer (CodeFree®, SD Biosensor, South Korea) for glucose measurement and this was recorded as glucose level at time $t_{-30}$. Afterwards, the rats were orally administered the corresponding treatment. After 30 mins, blood glucose levels of the rats were measured and recorded as blood glucose levels at time $t_0$. The rats received an oral dose of glucose (2 g/Kg body weight), and blood glucose levels further recorded at 30 60, 90, 120, and 240minutes.

**Determination of the Percentage Suppression Rate of the Postprandial Glycemic Peak:** This parameter evaluates the extent to which the treatments restrict the glycemic spike observed after administration of 2 g/kg of glucose. It is calculated as follows:

$$\%PS = \frac{[\text{Glu} + 30\text{control} - \text{Glu} + 30\text{test}]\text{x}100}{\text{Glu} + 30\text{control}}$$

Where %PS: percentage Suppression Rate of the Postprandial Glycemic Peak; $Glu+30_{control:}$ Blood glucose at $t_{30}$ of the negative control (distilled water); $Glu+30_{test}$: Blood glucose at $t_{30}$ of the test group being considered (ITM or reference drug).

**Determination of glycemia restoration time ($t_R$):** This parameter depicts the time after glucose administration where the fasting blood sugar is similar to the initial value. It was deducted directly from the glucose variation plot by extrapolation.

**Statistical analysis**

The Statistical Package for Social Science (SPSS) software version 26.0 for Windows was used to analyze the data obtained from this study. Means

of the different groups were compared with the one-factor ANOVA (Analysis of Variance) test. Results were expressed as the mean ± standard deviation and student's Newman Keuls test used to compare the means between two groups at 5% significance level.

**Ethical considerations**

The proposal including the standard operating procedures used in this research were reviewed and approved by the University of Buea Institutional Animal Care and Use Committee (UB-IACUC) and ethical clearance was obtained from the committee with reference number UB-IUCUC N° 05/2023.

**RESULTS**

**Extraction yield and phytochemical profile of the different plant materials**

The yield of lyophilised leaf extract of *A. conyzoides* was (5.99%), lower than *P. americana* (22.24%), and *M. indica* (28.05%).

Table 2 presents the main secondary metabolites of interest revealed in the different extracts of *M. indica, P. americana and A. conyzoides*, using semi-quantitative methods. Alkaloids, phenol, tannins, anthraquinones, antocyanins, tri-terpenes and flavonoids were found in both *M. indica* and *A. conyzoides* in varying proportions, while all phytochemicals except tannins were also present in *P. americana*

**Table 2:** Phytochemical constitution of different plant extracts (semi-quantitative analysis)

| | MI | AC | PA |
|---|---|---|---|
| Alkaloids | ++ | ++ | + |
| Phenol | + | ++ | + |
| Tannins | + | + | - |
| Anthraquinones | ++ | ++ | + |
| Anthocyanins | + | + | + |
| Tri-terpenes | + | + | + |
| Flavonoids | + | + | + |

+++: strongly positive; ++: moderately positive; +: weakly positive; -: negative. MI: *M. indica* lyophilized leaf water extract, PA: *P. americana* lyophilized leaf water extract, AC: *A. conyzoides* lyophilized leaf water extract.

Alkaloids, phenols, tannins, anthroquinones, anthocyanins, triterpenes and flavonoids were present in both *Mangifera indica* and *Ageratum conyzoides*, while *Persea americana* contained all the above phytochemicals except tannins. Quantification of some of the detected phytochemicals showed results summarized in Table 3 below.

**Table 3:** Quantification of metabolites of interest in the different plant materials

| | MI | AC | PA |
|---|---|---|---|
| Polyphenols (mgEAG/100g) | 17.24±16.60$^{a}$ | 26.38±12.35$^{a}$ | 11.44±8.59$^{a}$ |
| Alkaloids (µg Equi/mg of fraction) | 26.77±21.16$^{a}$ | 17.64±11.97$^{a}$ | 8.38±21.38$^{a}$ |
| Flavonoids (µg EC/mL of extract) | 128.44±.38$^{b}$ | 60.22±.38$^{a}$ | 128.44±.38$^{b}$ |
| Saponins (mg) | 3.51±.00$^{b}$ | 1.41±.00$^{a}$ | 0.51±.00$^{a}$ |

MI: *M. indica* lyophilized leaf water extract, PA: *P. americana* lyophilized leaf water extract, AC: *A. conyzoides* lyophilized leaf water extracts. On same row, the values with similar letters are statistically comparable with each other, unlike those carrying different letters

Upon quantification of polyphenols, alkaloids, flavonoids and saponins, the quantity of flavonoids in *A. conyzoides* was significantly lower ($p<0.05$) while the quantity of saponins and tannins in *M. indica* were significantly greater ($p<0.05$).

### *In vitro* antioxidant profile of the different plant materials

The antioxidant activity of lyophilized extracts were evaluated using the DPPH radical scavenging assay and Ferric reducing . The antioxidant activity is dependent on the hydrogen donating ability of the extracts. Figure 2 and Figure 3 show the dose response curves of DPPH radical scavanging activity and the Ferric reducing power activity of the extracts respectively.

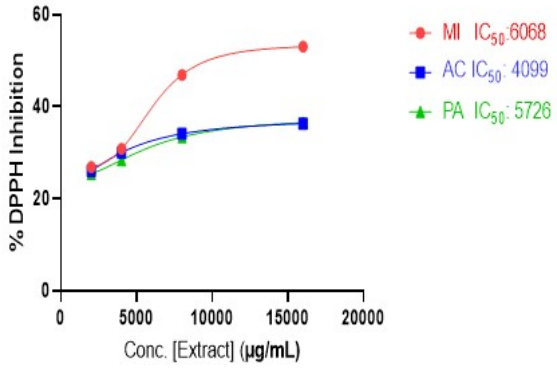


**Figure. 2. DPPH free radical scavenging potential of the different plant extracts**

MI: *M. indica* lyophilized leaf water extract, PA: *P. americana* lyophilized leaf water extract, AC: *A. conyzoides* lyophilized leaf water extracts.

There was a significant increase in the free radical scavenging activity of all three extracts MI, AC, and PA with respect to increasing concentrations, and this activity was depicted by the percentage inhibition of all extracts. However, AC had the least $IC_{50}$ value of 4099 µg/mL. Results obtained from comparing the % inhibition revealed there was no significant difference across the groups.

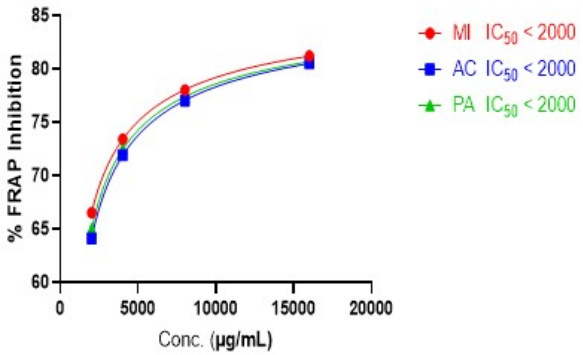


**Figure. 3. FRAP *i*activity of the different plant extracts**

MI: *M. indica* lyophilized leaf water extract, PA: *P. americana* lyophilized leaf water extract, AC: *A. conyzoides* lyophilized leaf water extracts.

All the extracts showed good Ferric reducing antioxidant power by a significant increase ($p<0.05$) in percentage inhibition with increasing concentration of extracts within the groups. At a concentration of 8000 µg/mL, the antioxidant activity of MI was significantly higher ($p<0.05$), compared to AC and PA. The $IC_{50}$ of all 3 extracts was found to be <2000 µg/mL

**Pre-formulation characteristics:** The pre-formulation characteristics of the different lyophilized extracts are presented in Table 4.

| Characteristics | MI | AC | PA |
|---|---|---|---|
| Appearance of the lyophilized powder | Brown, Solid powder | Dark-brown, Solid powder | Dark-brown, Lose powder |
| Appearance after drying at 40°C and spraying | Brown, lose powder | Dark-brown, lose powder | Dark brown, lose powder |

**Characteristics of the formulated capsules:** The invariability tests performed included residual moisture of the lyophilized powders after drying, the mass uniformity test, and Capsule disintegration time. These characteristics of the two formulated capsules are summarized in Table 5.

**Table 5.** Physicochemical characteristics of the different formulated capsules

| Characteristics | ITM-2 | ITM-1 |
|---|---|---|
| **Invariability tests characteristics** | | |
| Average mass per capsule | 0.99±0.041 | 0.97±0.007 |
| Disintegration time (min) on 5 capsules | 9.1±0.707 | ND |
| Residual moisture of the powder after drying (%) | 3.85±0.070 | 4.50±0.212 |
| **Mass uniformity test** | **Compliant** | **Compliant** |
| Average capsule weight | 0.99±0.041 | 0.970±0.007 |
| Average mass of powder per capsule: full capsule – empty capsule | 0.827±0.0142 | 0.808±0.031 |
| Deviation from theoretical weight | 0.005 | 0.006 |
| **Others** | | |
| Average dose | 25mg/kg/day | 25mg/kg/day |
| Number of capsules produced for the batch | 47 | 07 |

*ND: Not determined because of insufficient quantity.*

**Evaluation of cytotoxicity**

Cytotoxicity evaluation of both capsules was performed from 1000µg/mL to 7.8125 µg/mL against Normal African green monkey kidney epithelial (Vero) cells. The cells showed no significant decrease in cell viability, with a 50% cytotoxic concentration ($CC_{50}$) >1000 µg/mL

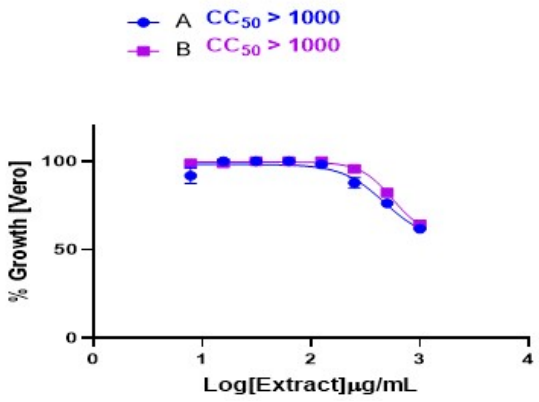


**Figure 4: Effects of ITM-1 and ITM-2 formulated capsules on Vero cell line viability**

A: ITM-2 (25 mg/kg), Capsules of *M. indica* lyophilized leaf water extract; B: ITM-1 (25 mg/kg), Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts

**Evaluation of acute toxicity**

Results obtained from the acute toxicity evaluation revealed that no toxic outcomes were observed upon administration of a dose 2000 mg/Kg body weight of either *M. indica* capsules or the capsule consisting a combination of *A. conyzoides, M. indica and P. Americana*.

**Effects of extracts on body weight in mice:** Generally, there was a steady increase in the body weight of the animals in treatment and control groups for both males and females, from day 0 through 7 to 14, with no statistically significant difference recorded when the control groups were compared to the treatment groups ($p<0.05$). Figure 5 and 6 below represents how the extracts affected the evolution of body weight in male and female mice.

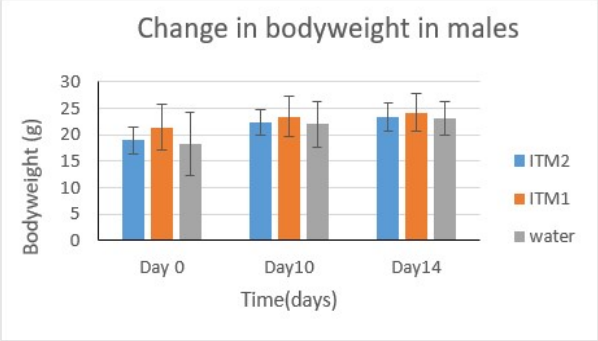


**Figure 5: Effects of extracts on body weight in female mice**

ITM-1 (25 mg/kg), Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts; ITM-2(25 mg/kg),Capsules of *M. indica* lyophilized leaf water extract

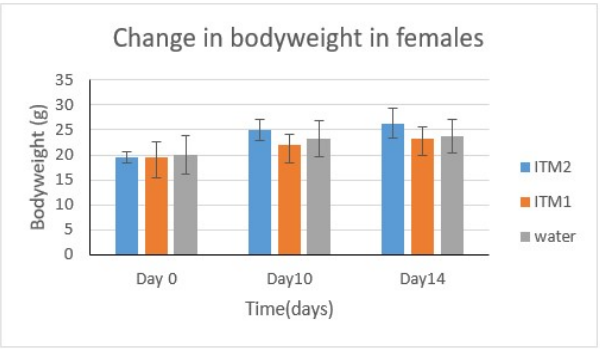


**Figure 6: Effects of extracts on body weight in female mice**

ITM-1 (25 mg/kg), Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts; ITM-2(25 mg/kg),Capsules of *M. indica* lyophilized leaf water extract;

**Effects of extracts on physical appearance and mortality:** Fur loss, change in fur texture, color or eye color was not observed in any of the treatment groups. There was also no evidence of nasal, buccal or genital secretions. Agility and ease of movement were also monitored and all the mice in both control and treatment groups responded to stimuli and moved with ease. There was a 100% survival rate of mice in all test and control groups as illustrated in figure 6 below.

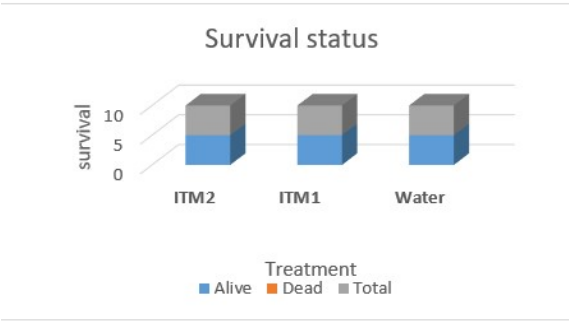


**Figure 7: Survival status of all mice (Both male and female)**

ITM-1 (25 mg/kg), Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts; ITM-2 (25 mg/kg), Capsules of *M. indica* lyophilized leaf water extract;

**Hypoglycemic and antihyperglycemic activity of capsules**

The OGTT was used to evaluate hypoglycemic and antihyperglycemic activity of the capsules in rats and the results presented in Table 6 and Figure 4. The ITM-2 capsule produced a significantly lower peak ($p<0.05$) at 30 and 60 min when compared to the negative control. The hypoglycemic effect of the polyherbal capsule ITM-1 drug was delayed, occurring after 60 mins, and persisted throughout the OGGT.

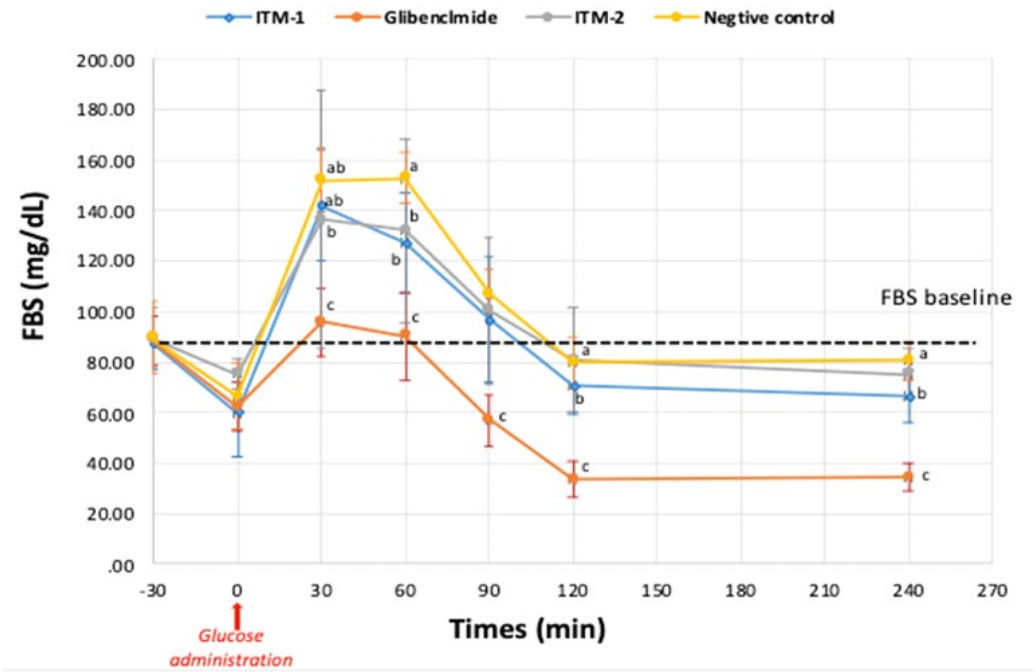


**Figure 8:** Evolution of glycaemia variation during OGTT

ITM-1 (25 mg/kg): Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana*lyophilized leaf water extracts; ITM-2(25 mg/kg):Capsules of *M. indica*lyophilized leaf water extract; Glib (Positive control): Glibenclamide (10 mg/kg); Water (Negative control): Distilled water (10 mL/Kg).

**Table 6:** Determination of percentage peak suppression and glucose restoration time

| Treatment | Peak suppression rate (%) | Restoration time (minute) |
|---|---|---|
| **ITM-2** | $26.58 \pm 52.03^{ab}$ | 110 |
| **ITM-1** | $9.37 \pm 17.79^{b}$ | 100 |
| **Glib** | $61.49 \pm 23.66^{a}$ | 60 |
| **Water** | N/A | 120 |

*Values with different letters are significantly different.*

ITM-2 (25mg/kg): Capsules of *M. indica* lyophilized leaf water extract; ITM-1 (25mg/kg): Capsules of combined *A. conyzoides*, *M. indica* leaves and *P. americana* lyophilized leaf water extracts; Glib (Positive control): Glibenclamide (10mg/kg); Water (Negative control): Distilled water (10mL/Kg).

## DISCUSSION

Teas from the plants *M. indica, P. americana* and *A. conyzoides* showed efficacy in type 2 DM rat model, with no significant acute toxicity (Zofou *et al.*, 2023), suggesting the use of these extracts for treatment of the metabolic disorder. This prompted the present work focused on development of standardized category 3 Improved Traditional Medicines (ITMs) from the traditional preparations, in order to guarantee accessibility to the treatment as well as standardization of doses regimen in patients.

Antioxidants are vital as free radical scavengers, reducing agents, and preventers of reactive oxygen species formation, thus preventing oxidative stress (Ashafa *et al.*, 2010). Diabetes mellitus leads to an increased production of reactive oxygen species, while reducing its scavenging, and this is usually caused by distortion in antioxidant enzymes, altered glutathione metabolism and increased ascorbic acid levels (Gaikwad *et al.*, 2014). As such, oxidative stress is apparent in the pathogenesis of diabetes mellitus and needs to be addressed (Ononamadu *et al.*, 2019). It was

therefore essential for the antioxidant activity of the lyophilized water extracts to be evaluated. The lyophilized extracts from the leaves of *M. indica, P. americana* and *A. conyzoides* showed dose dependent antioxidant activity in the DPPH and the FRAP assays, with higher extract concentrations having better outcomes in free radical destruction. For the DPPH assay, there was no statistically significant dose dependent scavenging activity across the different treatment groups, AC, MI and PA. Notwithstanding, AC had the lowest 50% inhibitory concentration of 4099 µg/mL. The $IC^{50}$ is inversely proportional to scavenging activity, thus AC shows more proton donating ability, serving as a better free radical scavenger. These results tie with those of Vakasari *et al.* (2022) who showed that the ethanol extract of *A. conyzoides* leaves had an antioxidant scavenging activity in a DPPH assay with an $IC^{50}$ of 153,63 µg/mL.

For the FRAP assay, all extracts showed good ability to reduce Iron (III) to Iron (II) with a unanimous $IC_{50}$ of <2000. However, *M. indica* showed a significantly higher ($P<0.05$) reducing power at all concentrations in the FRAP assay. Kigne *et al.* (2018) revealed that the hot water extracts of *M. indica* and *P. Americana* leaves are good sources of antioxidants. The high antioxidant activity can be associated to their flavonoids and phenolic contents (Kigne *et al.*, 2018). Phytochemicals such as flavonoids have experimental evidence of inhibiting reactive oxygen producing enzymes such as xanthine oxidase, Glutathione-S-transferase, lipoxygenase, NADH oxidase, thus inhibiting reactive oxygen production (Pietta, 2000). It is therefore important to prevent oxidative stress in diabetes as a major step towards limiting the progress and complications associated with this condition.

Capsules were formed from the lyophilized extracts, and their pre-formulation and formulation characteristics studied. The mass variation of capsules was in compliance with the standards of the European Pharmacopoeia 10th ed. which stipulates that, the mass of the capsules can vary up to 10% for capsules of less than 300mg and 7.5% for weights above 300mg. The evaluated capsules had a mean mass of $0.97\pm0.007$ and $0.99\pm0.041$ for ITM-1 and ITM-2, respectively. For the mass uniformity test, it is vital for the quantity of active extracts to be homogenous in all capsules to avoid variability of the administered dose (Ouedraogo *et al.*, 2021). The ITM-1 capsules had a deviation of 0.006 while ITM-2 had a minimum deviation of 0.005. Capsules were therefore compliant to the mass uniformity test. The average disintegration time of ITM-2 was $9.1\pm0.707$ mins which is less than the highest capsule disintegration time of 15 minutes (Stegemann *et al.*, 2014). Generally, the formulated capsules had good characteristics according to the recommendations of the European Pharmacopoeia 10th ed.

The OGTT was used to evaluate the acute effect of the drug formulations by assessing if the drugs improve glucose uptake and use, or optimizes glucose homeostasis. We therefore monitored the effect of the formulations on fasting blood sugar after a 30mins period and compared the time needed by the capsules to restore glucose levels back to normal after a post prandial glucose spike. The monoherbal capsule ITM-2 slightly dropped glucose levels 30 mins after its administration and significantly suppressed the post prandial peak in rats, compared to the negative control distilled water. These results emphasize the hypoglycemic and antihyperglycemic activity of *M. indica* capsules. Contrary to these results obtained with ITM-2, the combination capsules ITM-1 had a higher post prandial peak compared to the negative control and only restored glucose levels to normal after 100mins. These results were similar to those obtained by Zofou *et al.* (2023) in which there was

a significant reduction in the post prandial glucose peak in rats receiving monoherbal tea (MI) and the polyherbal tea (MI+AC+PA) sustained the postprandial peak. Here, ITM-1 polyherbal ITM had lower anti-hyperglycemic (postprandial peak suppression) effect but the initial glycemia was reached faster, as compared to the monoherbal preparation ITM-2. The antihyperglycemic and hypoglycemic activity of *M. indica* capsules could be justified that ITM-2 might act by reducing glucose absorption in the intestine as proposed by Aderibige *et al.* (2019) who obtained similar results when they evaluated the antihyperglycemic activity of water extracts of *M. indica*. Nyunai *et al.* (2009) reported that aqueous extract of *A. conyzoides* leaves at 200mg/kg and 300mg/kg showed statistically significant hypoglycaemic and antihyperglycaemic activities with a significant decrease in food, water intake and water excretion. Nyemb *et al.* (2009) also showed that the aqueous extracts of *A. conyzoides* at 200 mg/Kg and 300 mg/kg showed statistically significant hypoglycaemic and antihyperglycaemic activities. Similarly, for *P. americana* (Njateng *et al.*, 2018) in an *in vitro* study showed that methanol extract from leaves of *P. Americana* Mill strongly inhibits alpha-glucosidase, maltase-glucoamylase, aldose reductase and aldehyde reductase and showed almost no effect against beta-glucosidase. Also, Chimi *et al.* (2019) carried out an *in vivo* study where it was shown that the methanol and aqueous extracts of the leaves of *P. Americana* have high antidiabetic activities with 150 mg/Kg as the best dosage. These above results probably justify why these plants species are used in folk medicine for the treatment of diabetes, and suggest validity of the formulated antidiabetic drugs presented herein. However, the main goals of drug development are effectiveness but also safety.

Drug safety is one of the main aspects of disease therapy, which can play a major role in deciding which drug should be given to a patient considering the concept of benefit–risk balance. It is well known that all existing drugs can harm as well as help, safety is relative, with the difference between the effective dose and the dose that causes adverse/side effects referred to as the margin of safety. Owing to this knowledge, the formulated drugs were tested for safety *in vitro* and *in vivo*.

The cytotoxicity of capsules was measured by evaluating the percentage inhibition of Vero cells growth in the presence of the extracts. At the maximum concentration of 1000µg/mL, there was still a significant growth of cells for both capsules, indicating that the concentration of the extracts must be increased to obtain a sigmoidal growth response curve with results from a decrease in cell viability. These results were similar to those obtained by Ediriweera *et al.*(2006) who showed that extracts of *M. indica* showed less cytotoxicity to normal mammary epithelial cells.

Likewise, results obtained from *in vivo* acute toxicity testing in Balb/c mice did not reveal any toxic outcomes upon administration of a dose 2000mg/Kg body weight of either ITM-1 or ITM-2, with a 100% survival rate in both male and female mice and no altered behavioral patterns. Also, the high doses of ITM-1 and ITM-2 did not impair animal growth in terms of body weight increase. These results were similar to those obtained by Reddemen *et al.* (2019) who observed that there was lack of mortality and toxic changes upon administration of a maximum does of 2000 mg/Kg *Mangifera indica* extract containing 60% mangiferin to male and female Wistar rats. Likewise, the results are also relatable to those gotten by Nyunai *et al.* (2011). They assessed the acute toxicity of aqueous extract of *Ageratum conyzoides* in single doses of 3-13g/Kg and found that there were no changes in general behavior, no adverse effects and no mortality. Thus,

combining the 3 plant species to form ITM-2 did not induce any toxic effect. The absence of observable toxic outcomes from both the acute toxicity assay and the cytotoxicity assay of ITM-1 and ITM-2 capsules suggest that these ITMs could be used safely in diabetes management.

## CONCLUSION

Two category-2 improved traditional medicine drugs were formulated from 3 Cameroonian medicinal plants. The potential antidiabetic improved traditional medicines were rich in several plant secondary metabolites, showed significant antioxidant, and hypoglycemic properties. Both capsules named ITM-1 and ITM-2 were relatively safe following toxicity testing. Overall, these results suggest safe use of the formulated ITMs, and call for further toxicological investigations as well as elucidation of the sub-acute antidiabetic activity and mechanism of action.

## COMPETING INTERESTS

The authors declare that there is no conflict of interest.

## ACKNOWLEDGEMENTS

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