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Ethanolic extract of *Iwong* (*Ipomoea alba* L., Convolvulaceae) attenuates Adipogenesis in 3T3-L1 adipocytes

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Abstract
Several traditional medicinal plants have been investigated with respect to their anti-obesity potential. *Ipomoea alba* (common names: Iwong or Moon Wine) is used in Cameroonian traditional medicine as a laxative and anti-diabetic agent as well as to promote weight loss and improve breast milk quality. The goal of our study was to evaluate the effect of *Iwong* in cultured adipocytes. We assessed the effect of ethanolic extracts of *Iwong in vitro* on adipogenesis in the 3T3-L1 preadipocyte cell line. Treatment of 3T3-L1 cells with ethanolic extracts of *Iwong* significantly reduced lipid accumulation after 9 days. This reduction was associated with a significant decrease in glycerol-3-phosphate dehydrogenase (GPDH) activity and PPARγ and C/EBPα mRNA levels. The anti-adipogenic effect of Iwong was restricted to the early phases of adipogenesis. Our results indicate that *Iwong* contains natural substances potentially suitable for the treatment of obesity via inhibition of lipid accumulation.

Keywords: *Ipomoea alba*, *Iwong*, *Moon Wine*, Resveratrol, anti-adipogenic, 3T3-L1

1. Introduction

Obesity is a result of a prolonged imbalance between the levels of energy intake and energy expenditure [1]. Excessive energy is then stored as triacylglycerol in adipocytes. The consequence of lipid accumulation is an increase in adipose tissue mass which generally involves increases in fat cell size (hypertrophy) and in fat cell number (hyperplasia) [2]. Since the discovery of the capacity of adipocytes to synthesize and secrete leptin and other adipokines [3], adipose tissue is no longer considered as a simple fat storage tissue. Increase of body fat mass, particularly abdominal adiposity, is associated with increased risk of type 2 diabetes mellitus, hypertension, dyslipidaemia, cardiovascular disease and cancer [4] which is likely to be promoted by altered adipokine secretion patterns of hypertrophic adipocytes. The general health benefits of reducing fat mass in overweight and obese people are evident. In fact, improvements in obesity related metabolic dysfunctions are already observable with a reduction of 5-15% of initial body weight [5-7].

The decrease of adipose tissue mass involves a reduction of the number of adipocytes or preadipocytes through apoptosis, or a diminution of the size of fat cells by the mobilization of lipids in mature fat cells through lipolysis [8], or by the inhibition of lipid accumulation in maturing adipocytes [9].

Several pharmacological treatments have been elaborated with the objective to promote weight loss. Unfortunately, current pharmacotherapeutic remedies for the treatment of obesity and related metabolic disorders remain limited and ineffective [10]. Scientific investigations of medicinal plants are a key source for new discoveries with potential in obesity treatment and prevention [11]. There have been multiple claims for medicinal plants to exert anti-obesity actions of which some such as *Salacia reticulata, Panaxjaponicus, Momordica charantia* and *Dioscorea nipponica* are already in use for the prevention and the treatment of obesity [12-15].

The chances for success in medicinal plant research are certainly improved when the selection of a plant is based on its traditional use [16]. Therefore we conducted an ethno botanical survey in order to identify medicinal plants with weight loss properties as suggested by traditional healers in Cameroon. Based on this survey three medicinal plants were collected and their ethanolic extracts screened for anti-adipogenic action in preadipocyte culture. As an outcome of our ethno pharmacological survey as well as initial cell culture screening, *Ipomoea alba* (common Cameroonian name: *Iwong*; common English name: Moon Wine) was the most potent plant with little signs of toxicity and maximum effect on adipogenesis. *Iwong* is widely used in traditional medicine of Cameroon to facilitate weight loss, and as anti-diabetic remedy.

It is also given as a laxative and has the ability to improve the quality of breast milk. In India the same plant is used as a snake-bite remedy, whereas in Nigeria it is used against headaches [13]. It has been demonstrated that *Iwong* has non-addictive analgesic properties, inhibits respiratory bursts in leukocytes and scavenges oxygen-free radicals [14, 17]. *Iwong* is also eaten as a vegetable in Sierra-Leone and in China where young leaves and fleshy calyces are cooked in soups and stews or used as curries [17].

Our objective in the present study was to examine *in vitro* the biological activities of ethanolic extracts of *Iwong* in respect to cell toxicity and adipogenesis.

2. Material and Methods
2.1 Plant material
The plant *Ipomoea alba* (Convolvulaceae) was harvested twice in the region of Yaoundé-Cameroon, the first batch from April to June 2008 (batch A), the second batch two years later from September to October 2010 (batch B). Samples were deposited in the national herbarium of Cameroon for identification (n° 44695). The aerial part of the plant was air-dried for three weeks. The dried plant material was crushed to powder and stored at room temperature for further usage.

2.2 Chemical and reagents
Phosphate buffered saline solution (PBS; cat n° 18912-014) and Dulbecco's Modified Eagle Medium (DMEM; cat n° 41965-039) were purchased from Gibco. Fetal bovine serum (FBS; P4417-100TAB) was obtained from Biochrom AG, Streptomycin/penicillin (cat n°A2212), insulin (cat n° 050M8401), dexamethasone (cat n°2000039), and 3-isobutyl-1-methyl-xanthine (cat n° I5879-1G) and Oil red O (cat n° O0625) were from Sigma. Isopropanol (cat n° 6752.4), ethanol (cat n° T913.2), formaldehyde (Art n° 497901) and Tris/HCl (Art n° 9090.3) were purchased from Carl-Roth GmbH 2-Mercaptoethanol (cat n° M6250), NADH (cat n° N8129), DHAP (cat n°D7137) and Resveratrol (cat n° R5010) were from Sigma.

2.3 Preparation of the ethanolic extract
One kilogram of the powdered *Iwong* was incubated with 3000 ml of ethanol 70% at room temperature for 24 hours. The liquid extract was filtrated (Whatman paper number 1) and concentrated in a rotary evaporator (R-210, BUCHI Laboratory Equipment) with a final yield of 13.5%. Ethanolic extracts were stored at 4°C. For treatment of 3T3-L1 preadipocytes the extract was diluted in 0.1 % DMSO. During the course of the experiments the two batches of *Iwong* powder, batch A and batch B, were used for ethanolic extraction which differed in their efficacy as judged from cell culture experiments. Batch B was more potent (25-75 µg/mL) compared to the batch A (100-300 µg/mL) as an inhibitor of triglyceride accumulation in 3T3-L1 adipocytes.

2.4 Effect of *Iwong* on adipogenesis
3T3-L1 mouse embryo fibroblasts obtained from the American Type Culture Collection (Manassas, VA) were grown and passaged in a growth culture medium containing high glucose DMEM medium 10 % of FBS and 1% penicillin and streptomycin (10.000 kU/L). After 2 passages, cells were seeded in 6 or 12 wells tissue culture plates and grown to confluence in the growth culture medium. After 2 days of confluence the adipocyte differentiation was induced by standard culture medium supplemented with 5µg/ml insulin, 0.25 µM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX). Three days later the medium was removed and post-differentiated cells were cultured in a standard culture medium with 5 µg/mL insulin. Medium was then changed every 2 or 3 days for 9 days differentiation.

To evaluate the effect of *Iwong* on adipocyte differentiation, the ethanolic extract of *Iwong* was dissolved in DMSO and filtered with a syringe filter (Ø=0.2µm). Cells were treated either with DMSO 0.1 % or with different concentrations of *Iwong* ethanolic extract dissolved in DMSO 0.1 % during 9 days of adipocyte differentiation. For comparison, we assessed the effect of Resveratrol as a positive control. Cells were treated with DMSO 0.2 %, or with various concentrations of Resveratrol dissolved in DMSO 0.2 % during 9 days.

Moreover, to determine during which phase of adipogenesis 3T3-L1 cells are most responsive to *Iwong*, cells were treated during early (day 0-2), intermediate (day 3-5) and late (day 6-8) phases of adipogenesis with *Iwong* ethanolic extract. All cells were exposed to 0.1% DMSO if not treated with *Iwong*. Medium was changed every three days. At day 8 of differentiation cells were stained with Oil Red O and amount of lipid accumulation was quantified as described above.

2.5 Cell Viability Assay
The cytotoxicity of *Iwong* was determined at day 3, 5 and 8 of differentiation using a commercial test system, according to the protocol provided by supplier (CytoTox-Glo kit, Promega cat # G9291). Luminescence was recorded with a luminometer (Sirius, Berthold Detection Systems GmbH, Germany).

2.6 Oil-Red-O (ORO) staining and lipid quantification
Oil-Red-O staining and lipid quantification were performed as described previously [16]. Briefly, dye stock solution was prepared with 0.5 g of ORO dissolved in 100 ml of isopropanol 99 % and left at room temperature for 24 hours. The stock solution was then filtered and mixed with distilled water (2v:3v) to prepare the working solution. The working solution was then filtered once again.

To stain cells, the medium of each well was aspirated. Cells were washed once with PBS and fixed with 3.7 % formaldehyde. After one hour the remaining formaldehyde was discarded and cells were incubated with the working dye solution for one hour. Cells were then immediately rinsed once with PBS.

For lipid quantification, the dye was extracted with isopropanol for 15 min at room temperature, and the absorbance was read with a spectrophotometer (Tecan infinite M200, Austria) at 492 nm. Each experiment was repeated 3 times in triplicate for every concentration of *Iwong*, Resveratrol, and the vehicle (DMSO).

2.7 Glycerol-3-Phosphate Dehydrogenase (GPDH) Activity
3T3-L1 adipocytes were differentiated in 6 well plates and treated either with *Iwong*ethanolic extract or DMSO 0.1 %. After 8 days of differentiation cells were washed twice with ice-cold PBS and lysed with a buffer containing 0.05 M Tris/HCl, 1 mM EDTA and 1 mM 2-mercaptoethanol pH 7.4. GPDH activity was determined with a spectrophotometric method by measuring the depletion of nicotinamide adenine dinucleotide (NADH) during GPDH-catalyzed reduction of dihydroxyacetone-3-phosphate (DHAP) at 340 nm [18]. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, cat n° 500-0116) with

bovine serum albumin as a standard. The enzyme activity was expressed as units per mg protein. All measurements were performed in triplicate for each treatment and repeated in three independent cell culture experiments.

2.8 Determination of glycerol release
Preadipocytes were differentiated to mature adipocytes for 14 days in 12 well plates; the medium was changed every two days during the entire differentiation period. Mature adipocytes were then incubated in the presence of *Iwong* ethanolic extract (50 and 75 µg/mL), isoproterenol (1µM) or DMSO (0.1%) for 24h. The free glycerol in the medium was assayed enzymatically using a commercial kit according the manual (r-biopharm, Germany, cat n° 10148270035). The experiment was repeated three times in triplicate.

2.9 Gene expression analysis
Post-confluent preadipocytes were treated with *Iwong* ethanolic extracts during the early phase of adipogenesis (day 0-2), and then cultured in DMEM with

insulin until the end of the differentiation period. Cells were harvested at day 8 of differentiation for RNA extraction according to the provider's instructions (Total RNA Isolation System, Promega, Madison, USA, cat n° Z3105). RNA concentration was determined spectrophotometrically (Nano quant plate, Tecan, Salzburg, Austria) and stored at - 80°C. Denaturing agarose gel electrophoresis was performed to check the quality of the RNA. The cDNA was synthesized by reverse transcription (Quanti Tect, Qiagen, Hilden, Germany, cat n° 205314). The transcript levels of the transcription factors PPARγ and C/EBPα were measured by quantitative PCR (qPCR) and normalized to GAPDH expression. Primer sequences used for the qPCR are depicted in table 1.

2.10 Statistical analysis
One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were performed to analyze all data. Results are expressed as mean ± SD. $P<0.05$ was set as level of significance. Statistical analysis was performed using a commercial software package (Sigma Stat 3.0).

Table 1: Primer sequences used for gene expression analysis

Gene	Orientation	Primer sequence
PPARγ	Forward	TTTTCAAGGGTGCCAGTTTC
	Reverse	AATCCTTGGCCCTCTGAGAT
C/EBPα	Forward	TGCTGG AGTTGACCAGTGAC
	Reverse	AAACCATCCTCTGGGTCTCC
GAPDH	Forward	TGTGTCCGTCGTGGATCTGA
	Reverse	CCTGCTTCACCACCTTCTTGAT

3. Results
3.1 Cytotoxicity of ethanolic *Iwong* extract
To assess cytotoxicity, 3T3-L1 Preadipoctyes were treated with four different doses of ethanolic *Iwong* extracts for 3, 5 and 8 days during adipogenesis. In general, the cytotoxicity read out was highest on day 3 after induction of adipogenesis, indicating that more cells died in the early post-confluent

phase, whereas intermediate and late phases of adipogenesis a smaller fraction of the cells died. *Iwong* had no cytotoxic effect on day 3 after induction, but at days 5 and 8 there was a weak trend towards cytotoxic effects with increasing *Iwong* concentrations, which attained significance at 300µg/mL (Fig. 1A).
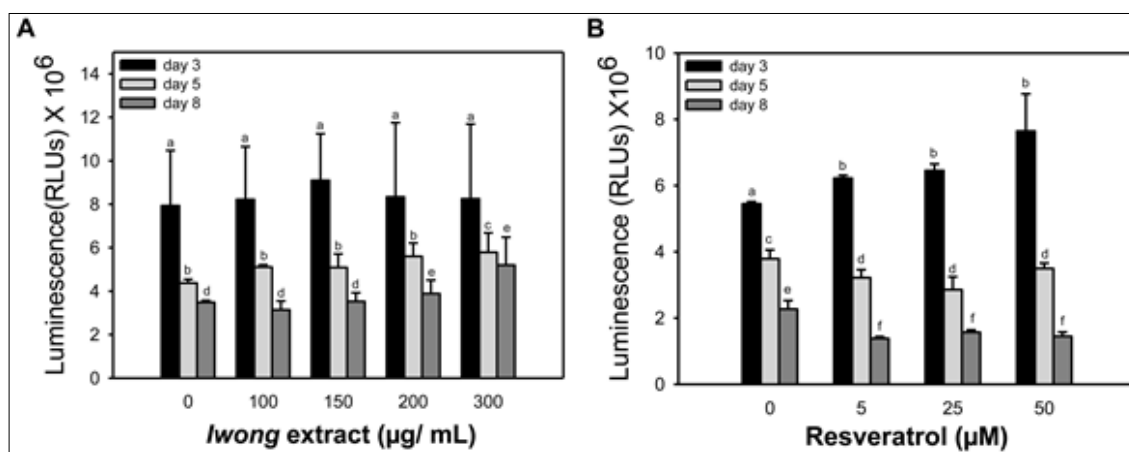

Fig 1: Cytotoxicity of ethanolic extract of Iwong and resveratrol. (A) Two-days post confluent 3T3-L1 cells were differentiated according to the MDI protocol in the presence of 100µg/ml, 150µg/ml, 200µg/ml and 300µg/ml of Iwong. The cytotoxicity of the extract was determined after 3, 5 and 8 days of incubation. (B) Resveratrol was used as positive control, and cells were treated either with 5, 25 and 50 µM of resveratrol or with DMSO 0.2%. As indicate the results are means of 3 wells ± SD of 3 independent experiments. Values with unlike letters are significantly different.

Opposite results were obtained with Resveratrol (Fig. 1B). A significant increase on cytotoxicity was noticed after 3 days of the treatment ($p<0.05$), whereas at the intermediate (day 5) and late (day 8) phases of treatment, a significant decrease of cytotoxicity was observed on all cells treated with Resveratrol ($p<0.05$).

3.2 *Iwong* attenuates lipid accumulation
The effect of *Iwong* on neutral lipid accumulation in 3T3-L1 was evaluated using Oil Red-O dye staining which indicates intracellular triglyceride and cholesterol accumulation. The staining intensity is positively correlated with neutral lipid accumulation. Oil Red-O staining can be taken as a surrogate measure for adipogenesis (Fig. 2A).
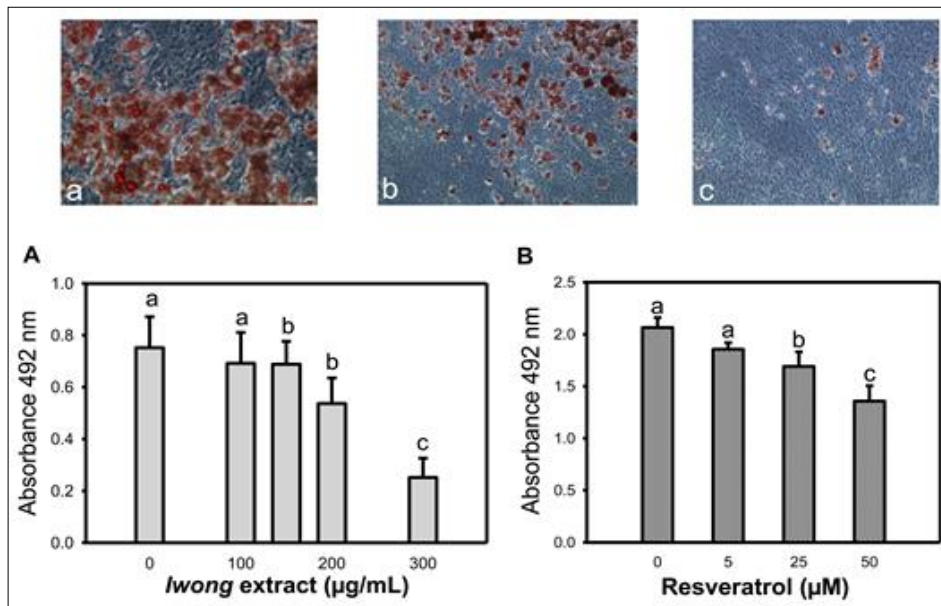
Fig 2: *Effect of ethanolic extract of Iwong and Resveratrol on lipid accumulation*. Two days post confluent 3T3-L1 preadipocytes were differentiated with MDI in the presence either of several concentrations of the ethanolic extracts of *Iwong* or DMSO 0.1%. After 9 days cells were stained with Oil-Red-O and photographed at magnitude x100. Panels a, b and c display cells treated with DMSO 0.1%, 200µg/ml, and 300µg/ml, respectively. (A) Oil-Red-O was extracted by isopropanol and quantified spectrophotometrically at 492 nm. (B) Cells were also treated with Resveratrol 5µM, 25µM and 50µM or DMSO 0.2%. At day 9 of differentiation cells were stained by Oil-Red-O, which was extracted by isopropanol for lipid quantification. The amount of lipid was read spectrophotometrically at 492 nm in triplicates. Each experiment was repeated 3 times. Data are expressed as means ± SD, and values with unlike letters are significantly different.

3T3-L1 preadipocytes cultured in the presence of *Iwong* showed decreased Oil-Red O staining as compared to DMSO control, and thus decreased lipid accumulation. This effect was detectable at an *Iwong* extract concentration of 150 µg/ml. Maximum inhibition was observed with 300 µg/ml (Fig. 2B). A fresh ethanolic *Iwong* extract prepared from the second batch of plant material was also tested (batch B). Results obtained showed that this new extract was more potent, and the maximal inhibitory effect was observed at an extract concentration of 75 µg/ml (data not shown) compared to 300 µg/ml for the batch A.

Resveratrol, a flavonoid known for its anti-adipogenic effect was used as positive control. Post-confluent 3T3-L1 Preadipocytes were differentiated for 9 days in the presence of DMSO 0.2% or three concentrations of Resveratrol (5µM, 25µM and 50µM) and lipid accumulation was assessed. Resveratrol diminished lipid accumulation in a dose dependent manner attaining statistical significance at 25µM and 50µM ($p<0.001$) (Fig. 2B).

3.3 Inhibition of GPDH activity by *Iwong*

The enzyme GPDH occupies a central position in the pathway of triglyceride synthesis and is an established marker of adipogenesis. Enzyme activity of GPDH was strongly reduced after 9 days of chronic *Iwong* treatment in a dose dependant manner (Fig. 3A). *Iwong* significantly reduced GPDH activity at the concentrations 150 µg/mL ($p<0.05$), 200 µg/mL ($p<0.01$) and 300 µg/mL ($p<0.005$).
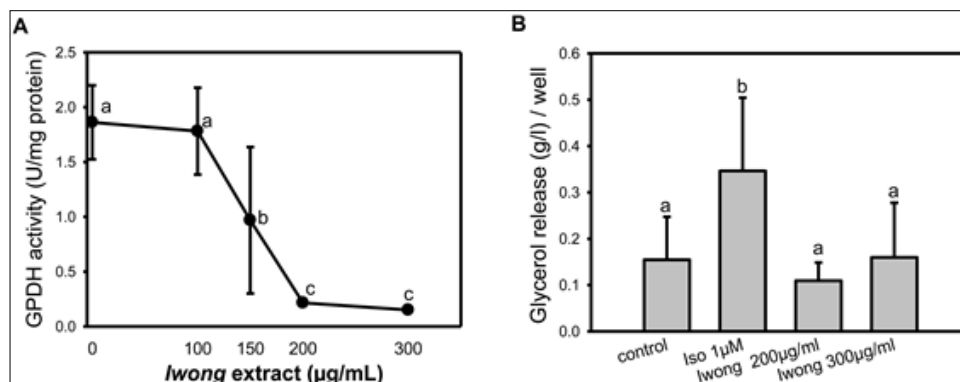

Fig 3: *Effect of ethanolic extract of Iwong on GPDH activity and glycerol release*. (A) GPDH activity was measured after 9 days of treatment with different doses of Iwong extract. (B)To assess the effect of Iwong extract on lipolysis, post confluent preadipocytes were differentiated for 14 days and then treated for 24h with 200 and 300µg/ml of *Iwong*, DMSO 0.1% (vehicle), or 1µM isoproterenol (Iso) as a positive control. Glycerol release into the medium was determined spectrophotometrically at 340nm. Each experiment was performed in triplicates and repeated at least 3 times. Data are expressed as means ± SD, and values with unlike letters are significantly different.

The effect on glycerol release was also tested in 3T3-L1 cells in the presence of *Iwong* (200 and 300µg/ml), isoproterenol (1µM) or DMSO (0.1%) for 24h. The ethanolic extract of *Iwong* had no effect on glycerol release compared to isoproterenol (1µM) used as positive control, where a significant ($p<0.05$) difference was observed, suggesting that *Iwong* did not affect lipolysis (Fig. 3B).

3.4 Inhibitory activity of *Iwong* is restricted to the early phase of adipogenesis

Treatment of 3T3-L1 preadipocytes during different phases of adipogenesis demonstrated that the presence of *Iwong* during the first three days (day 0-2) after induction of differentiation is fully sufficient to reduce lipid accumulation ($p<0.05$) to the same extent as observed for chronic treatment (day 0-8). Notably, *Iwong* exposure during intermediate (day 3-5) and late (day 6-8) phases of adipogenesis had no effect on lipid accumulation (Fig. 4).
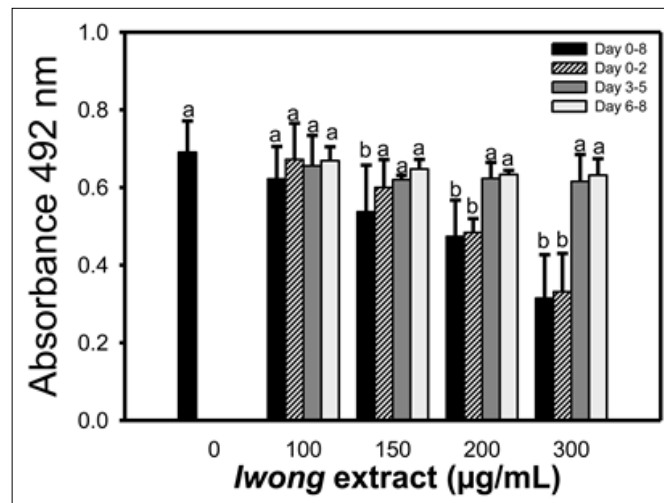

Fig 4: *Effect of ethanolic extract of Iwong on lipid accumulation in 3T3-L1 cells in different stages of differentiation.*3T3-L1 preadipocytes were proliferated for two days; the differentiation of post-confluent preadipocytes was induced by standard adipogenic medium to initiate adipogenesis in the presence of DMSO 0.1 % or 100, 150, 200 and 300 µg/ml of *Iwong*. The ethanolic extract of *Iwong* was added at three phases of the differentiation process from either day 0 to 2 (early phase), 3 to 5 (intermediate phase), 6 to 8 (late phase) and 0 to 8 (chronic treatment). Data are expressed as means ($n =3$) ± SD, and values with unlike letters are significantly different.

3.5 *Iwong* inhibits expression of key adipogenic transcription factors

To obtain first insight into the molecular mechanisms of *Iwong* action, the transcript levels of key adipogenic transcription factors were assessed in 3T3-L1 preadipocytes exposed to *Iwong*. These experiments were conducted with batch B of fresh *Iwong* extracts exhibiting a higher inhibitory effect on lipid accumulation (see Oil Red O section). Thus, concentrations in the range of 25 – 75 µg/ml were chosen. We observed a significant decrease of PPARγ and C/EBPα mRNA levels in cells treated with the ethanolic extract of *Iwong* at concentrations of 50 and 75 µg/ml (Fig. 5).
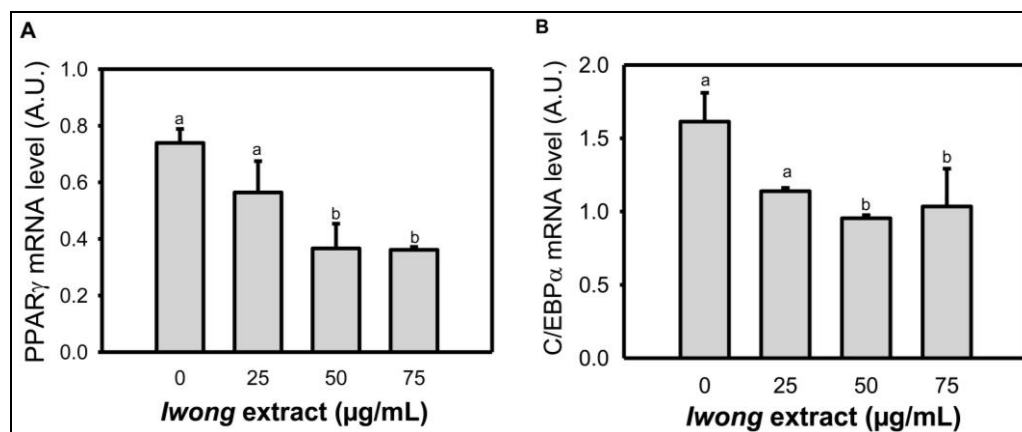

Fig 5: *Effect of ethanolic extract of Iwong on the mRNA level of two key transcription factors during 3T3-L1 adipogenesis.* Post confluent 3T3-L1 preadipocytes were treated with DMSO 0.1% or different concentrations of *Iwong* (25-75µg/ml) from day 0 to day 2 and with insulin present only from day 3 to day 8. Cells were harvested at day 8. The expression levels of mRNA of PPAR☐, C/EBP☐ and GAPDH were measured by quantitative PCR (qPCR). Values normalized by GAPDH and expressed inarbitrary unit (U.A.) are means ± S.D of three different experiments of PPARγ (A) and C/EBPα (B). Data with unlike letters are significantly different.

4. Discussion

Several medicinal plants with anti-obesity properties are known [19]. The activation of lipolysis, the mitigation of triglyceride synthesis as well as the inhibition of adipocyte differentiation are proposed strategies to treat obesity [10]. This study aimed to elucidate the effects of ethanolic extracts of *Iwong* on adipogenesis with respect to triglyceride synthesis, lipolysis and the expression of adipogenic transcription factors. Resveratrol, a flavonoid found in red wines and grape juice, was implemented as a positive control, as it inhibits lipid accumulation and key transcription factors involved in the adipogenic process [20]. Our experiments were carried out *in vitro* in the 3T3-L1 preadipocyte cell line, which is one of the best characterized models for studying the conversion of preadipocytes into adipocytes [21] and has been widely used to study the molecular mechanisms of the adipogenic program [22].

The ethanolic extract of *Iwong* significantly reduced lipid

accumulation and GPDH activity in a dose dependent manner, while not affecting lipolysis. In respect to lipid accumulation, the inhibitory effects of *Iwong* and Resveratrol implicated as positive control were comparable. In addition, the presence of *Iwong* ethanolic extract during the early (day 0-2) stage of differentiation significantly prevented fat storage in 3T3-L1 to the same extent as the chronic treatment (day 0 to 8). No effect was observed when the plant extract was added at the intermediate (day 3 to 5) or late (day 6 to 8) stages. These results clearly demonstrate that *Iwong* interferes with early events in the process of adipocyte differentiation resulting in the inhibition of triglyceride synthesis. Notably, a similar inhibitory effect on lipid accumulation at the early stage of differentiation as observed here with Iwong has also been reported in 3T3-L1 adipocytes treated with Esculetin [23].

The development of mature fat cells from preadipocytes is a complex process characterized by growth arrest and clonal expansion followed by changes in cell morphology, hormone sensitivity, gene expression and lipid storage [2]. Several transcription factors induce and maintain adipogenesis by changes in their gene expression levels [24]. We consequently investigated the effect of *Iwong* ethanolic extract on gene expression of PPARγ and C/EBPα, two transcription factors crucial for adipogenesis. These transcriptional regulators of adipocyte differentiation act synergistically to generate fully differentiated adipocytes responsive to insulin. Our results reveal a decrease of PPARγ and C/EBPα mRNA levels, suggesting that the anti-adipogenic action of *Iwongis* conveyed by down regulation of the transcription factor cascade upstream of PPARγ and C/EBPα.

This effect is in line with several other studies demonstrating that natural plant compounds such as Resveratrol, EGCG, Esculetin, Berberine and Guggulsterone inhibit lipid accumulation in 3T3-L1 cells by downregulating PPARγ and C/EBPα gene expression [25]. Our future work will aim at identifying in ethanolic extracts of *Iwong* the bioactive compound(s) responsible of this anti-adipogenic effect.

5. Conclusion
Taken together, our results demonstrate that *Iwong* plays a role in adipogenesis by inhibiting triglyceride accumulation in the early phase of adipogenesis. Thus, *Iwong* is a candidate medicinal plant with potential in the prevention and/or the treatment of obesity that requires further investigation in *in vivo* studies.

5.1 List of abbreviations: ALT: Alanine Transaminase, AST: Aspartate Transaminase, C/EBPα: CCAAT-enhancer-binding proteins alpha, DHAP: dihydroxyacetone-phosphate, DMEM: Dulbecco's modified Eagle's medium, DMSO: Dimethyl sulfoxide, EDTA: Ethylenediaminetetraacetic acid, FBS: Fetal Bovine Serum, GPDH: Glycerol-3-phosphate dehydrogenase, IBMX: 3-isobutyl-1-methylxanthine, NADH: nicotinamide adenine dinucleotide, ORO: Oil Red O, PCR: Polymerase Chain Reaction, PPARγ: Peroxysome proliferator-activated receptor alpha and qPCR: quantitative Polymerase Chain Reaction

5.2 Competing interests: The authors declare no conflict of interest.

6. Acknowledgements

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