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Balde ES
(1) Department of Pharmacy, FMPOS University Gamal Abdel Nasser Conakry Guinée
(2) Center for Research and Valorization of Medicinals Plants Dubréka Guinea

Camara AK
Department of Pharmacy, FMPOS University Gamal Abdel Nasser Conakry Guinée

Traoré MS
(1) Department of Pharmacy, FMPOS University Gamal Abdel Nasser Conakry Guinée, Guinea
(2) Center for Research and Valorization of Medicinals Plants Dubréka Guinea

Baldé NM
Endocrinology and Diabetology Unit, CHU Donka Conakry Guinea

Megalizzi V
Laboratory of Toxicology, Faculty of Pharmacy ULB Brussel Belgium

Pieters L
Natural Products and Food Research and Analysis, Department of Pharmaceutical Sciences, University of Antwerp, Belgium

Balde AM
(1) Department of Pharmacy, FMPOS University Gamal Abdel Nasser Conakry Guinée
(2) Center for Research and Valorization of Medicinals Plants Dubréka Guinea

Correspondence
Balde ES
(1) Department of Pharmacy, FMPOS University Gamal Abdel Nasser Conakry Guinée
(2) Center for Research and Valorization of Medicinals Plants Dubréka Guinea

The hypoglycemic and cytotoxic activity of the leave extract of *Combretum glutinosum* Perr ex DC

Balde ES, Camara AK, Traoré MS, Baldé NM, Megalizzi V, Pieters L and Balde AM

Abstract
Combretum glutinosum is widely used in Guinean traditional medicine in the treatment of various diseases such as diabetes and cancer. Aiming to find out the potential of *C. glutinosum* antidiabetic and cytotoxicity, the extracts were tested on the Han Wistar rat and the cytotoxicity on cancerous cell lines. Polar extracts of *C. glutinosum* normalized the glycaemia level 2H after intra peritoneal glucose injection in rat. Apolar extract of *C. glutinosum* was cytotoxic against cancer cell lines [$IC_{50} \leq 10\mu g/ml$]. No cytotoxic effet was reported with semi polar and polar extracts. Bioguided fractionation of SbDF conducted to isolation the compounds 5 hydroxy-7-4'-dimethoxy flavone, lupenone and lupeol. Data in the literature confirms the anticancer and antidiabetic activity of these compounds. Based on our results, *C. glutinosum* could be therefore investigated for new antidiabetic or anticancer drug.

Keywords: Hypoglycemic and cytotoxic activity, *Combretum glutinosum*

Introduction
As major public health problem, cancer and diabetes are the leading global cause of death over the world. Unfortunately, their prevalence continues to increase. In 2018, around 18.1 million new cancer cases and 9.6 million cancer deaths have been worldwide recorded. In 2017, although half of all people living with diabetes are undiagnosed, a global estimation of 451 million people has been described [1]. These figures were expected to rise to 23.6 million by 2030 or to 693 million by 2045 for cancers or diabetes, respectively [2].
Such prevalence has been rising as well in rich countries as in less developed ones including Sub-Saharan Africa. In Guinea, the prevalence of diabetes was estimated to 3.5 - 6.5% [3, 4] and that of cancer is still statistically undetermined. The Guinean traditional management of these non-communicable diseases are mainly based on the use of plant species from different botanical families [5, 6]. Among these, the leaves of Combretum glutinosum are used for the treatment of diabetes, breast wound healing, urinary infections…
Recent data have suggested that DT2 is associated with the development of cancers such as renal, esophageal, pancreatic, and breast cancer [7–11].
Moreover, hyperglycemia and hyperinsulinemia have been described as cancer promoters via their lipotoxicity, glucotoxicity and oxidative stress effects [12].
Since the use of metformin and thiazolidinediones was associated with a low risk while that of insulin, sulfonylureas and alpha glucosidase inhibitor was linked with an increased risk [13, 14], the relationship between the antidiabetic medications and the incidence of cancers has been of interest.
Based on these possible relationships between cancer and diabetes along with the traditional use of *C. glutinosum*, the hypoglycemic and cytotoxic activities of the plant extracts and fractions were investigated.

Materials and Methods
Preparation of plants extract
Extract for biologicals tests
Extract for anti-hyperglycaemic activity
20g of powdered leaves were treated by maceration for 24h either with 200ml of methanol or 300ml of distilled water. The extracts were filtered and evaporated in vacuum to give 5.4g of methanolic dry extract (MeSb) or lyophilised to give 4.2g of lyophilisat (AqESb).

Extract for Cytotoxic assay
The dried and milled leaves [50 g] were extracted by maceration with methanol [300 ml] at room temperature, for 24h. After filtration and evaporation of the solvent under reduced

pressure, the residual solution was diluted in H_2O to afford an aqueous solution (150ml), which was partitioned successively with dichloromethane (5×150 ml), ethyl acetate (5×150 ml), to afford dried SbDF (1.2 g) and SbEAF (1.3 g). The residual aqueous solution was lyophylised to afford AqESb (1.8 g).

Drug preparation:

Glibenclamide (Sigma-Aldrich, CAS N° 10238–21–8) was used as the test substance for *in vivo* test. Freshly solutions of aqueous and methanolic extracts (300 mg/kg) and glibenclamide (4 mg/kg) in 0,05N NaOH solution were prepared. The final pH of these solutions was between 7 and 8.

Animal model

The healthy male Wistar rats of 191 - 296 g weight range and 8 - 12 weeks were housed in individual metabolic cages with free access to food and water. All animals used in experiment were randomly selected and classified into four groups before beginning of experimental investigation. The animal grouping is as follows:

Group I: Normal rats treated with distilled water

Group II: Positive control Normal rats treated with glibenclamide 4 mg/kg

Group III: Normal rats treated with aqueous extract 300 mg/kg

Group IV: Normal rats treated with methanolic extract 300 mg/kg

Animal experimentations were conducted at the University of Mons [Belgium], according to the local ethical committee of the Institution.

Cell culture

Five cancer cell line A549 non-small-cell lung cancer (NSCLC), prostate carcinoma prostate (PC3), glioblastoma (U373, Hs683) and Kaka were used. All cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in minimum essential media (MEM) supplemented with 5 % or 10 % fetal calf serum (FCS), 20 mM l - glutamine, 2 % penicillin – streptomycin, and 0.2 % gentamicin. Cell media and all supplements were obtained from Gibco BRL (Gibco-Invitrogen, Merelbeke, Belgium).

Experimental design

Glucose challenge experiments:

All drugs except glucose were administered in a dose volume of 5ml/kg per os. For the glucose challenge test, glucose was given a dose volume 2ml/kg by subcutaneous injection. D-glucose was dissolved in saline solution (0, 9% sodium chloride) for challenge experiments.

An intra-peritoneal glucose tolerance test (IPGTT) is used to assess the body's ability to metabolize glucose. The experiment consisted of four groups of rats normal healthy male (4rats/group) to evaluate hyperglycaemia. Distilled water, glibenclamide aqueous extracts, and methanolic extract were administered per os to group 1-4 respectively. Each group received subcutaneous injection of glucose.

At the initial stage (t0), the level of glycaemia was measured before any administration. The rats are stuffed respectively with distilled water, glibenclamide, aqueous and methanolic extracts 30 min before the glucose solution is injected through subcutaneous way (1 g/kg of body weight). The glycaemia was measured 5 min (t5), 15min (t15), 30 min (t30) and 120 min (t120) after injection of glucose challenge.

Cytotoxicity evaluation

The efficacy of *C. glutinosum* extracts on the inhibition of the growth of cancer cells *in vitro* was investigated using the colorimetric 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide assay (MTT) (Sigma–Aldrich, Belgium) [15]. Cancer cells were incubated for 72 h in the presence or the absence of *C. glutinosum* extract fractions that ranged in concentration from 10^{-2} to 10^{2} µg/ml using an increase in concentrations. The IC_{50} values were determined from the extract dose versus cancer cell growth curves. All experiments were carried out in duplicate on A549, PC3, U373, Hs683 and kaka cells using 6 wells per treatment condition.

Extraction and separation

The dried and milled leaves (500g) were extracted by maceration with methanol (3 x 2000 ml) at room temperature for 24h and operation was repeated for 3 days. After filtration and evaporation of the solvent under reduced pressure, the residual solution was diluted in H_2O (400ml), and then partitioned in turn with dichloromethane (5×400 ml), to afford dried dichloromethane (20 g) extracts.

The extract SbDF extract (10g) which possessed a good cytotoxicity was fractioned by an open colunn chromatography over silica gel 60–200 mesh, using a CH_2Cl_2/MeOH eluant gradient polarity. Eluents fractions were monitored using pre-coated silica plates (TLC silica gel 60F254 Merck) with a Toluene/Acetone/Formic acid (15/14/3) mobile. This same operation was realized as well for the fraction (SbDF3) as their sub fraction.

Data analysis

The glycaemia values were presented by boxplot, the median value of glycaemia was compared by the W Kendall test and the significance was fixed at $p = 0.05$

Results and Discussion

Anti-hyperglycaemic effect

The glycemic levels of all the tested rats before any treatment were 0.95 - 1.62 g/l with a median of 1.1 - 1.31 g/l. These values were considered normal for such category of Han Wistar rats as previously described (1.23 ± 28 g/l).

As shown in Figure 1, both the aqueous and methanol extracts reduced the blood sugar level in normal Wistar rats. At T15 we observed the maximum value of glycemic level for all groups and ranged between 1.4 and 2.1 g/l, but with a minimum rate of elevation for glibenclamide and *C. glutinosum* aqueous extract.

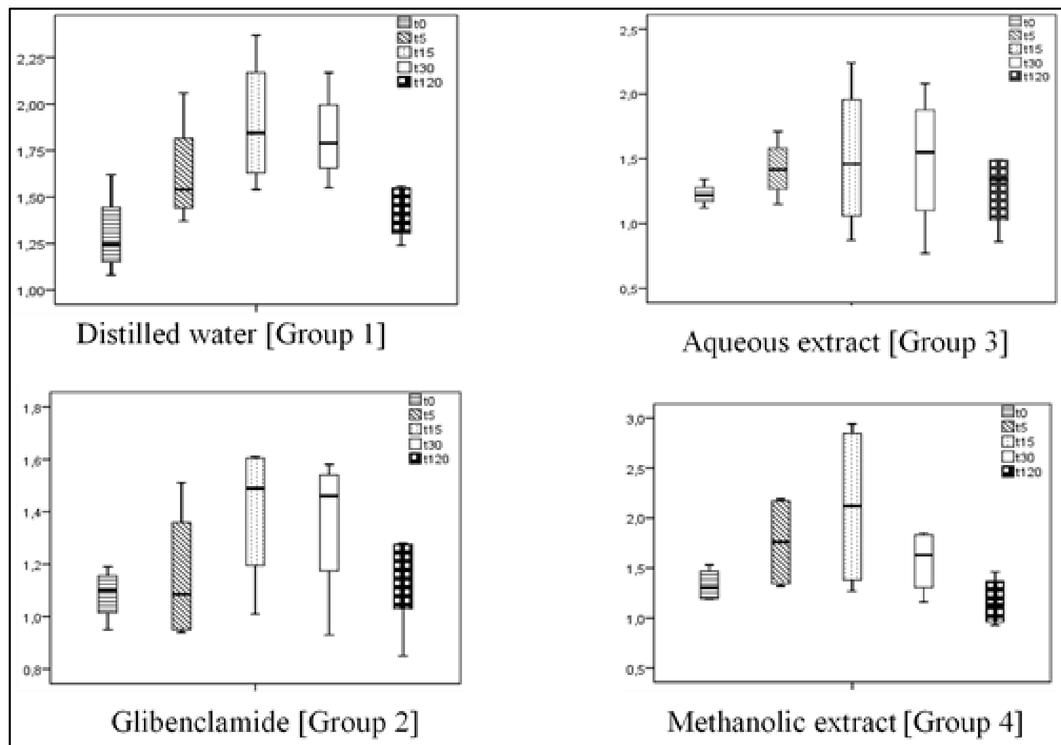

Fig 1: Acute effect of differents treatment on glucose tolerance [1g / kg] in the four groups

Excepted for the group1, the glycaemia value regression started at time T30 and no significant changes of blood glucose levels were observed in normal-glycemic rats treated with *C. glutinosum* extracts or glibenclamide during the 2 h of the experimental period. Conversely, in the group treated with distilled water, the changing in the blood glucose level during the experience was significant [p = 0.008]. But the decrease in glucose level was greater in the groups treated with Glibenclamide and the *C. glutinosum* aqueous extract.

From the combretaceae genus, previous ethnopharmacological studies have demonstrated the antidiabetic potency of *C. lanceolatum, C. molle,* and *C. micranthum* [16-19]. A Long-term daily administrations of *C. decandrum* extract at decreased fasting blood glucose in streptozotocin-induced diabetic rats [20].

Regarding the similarity of blood glucose lowering effects of *C. glutinosum* extracts [aqueous and methanolic] and Glibenclamide, and the improvement in glucose tolerance by aqueous extract of *C. glutinosum,* could probably stimulated the insulin secretion from pancreatic β-cells and interfered with the insulin receptor pathway.

Combretum species are widely used in traditional medicine and is also credited with broad pharmacological properties [21-24]. Anyway, this is the first report of the anti-hyperglycemic effect of *C. glutinosum*.

In vitro cancer cell growth inhibitory assay

The polar extract of *C. glutinosum* was partitioned by an apolar and semi polar solvent and they tested for their potential inhibition of cancer cell growth. As shown in table 1, all the tested cancer lines were sensible to the *C. glutinosum* leaf extracts. This activity seems to be closely related to the degree of polarity, the dichloromethane extract (SbDF) being the most active followed by the extract with ethyl acetate (SbAEF) while the polar extract (SbMF) remains the least active. The cytotoxic activity of the apolar SbDF was the widest and pronounced effect against adenocarcinomic human alveolar basal epithelial cells (A549), glioblastoma cell (U373; Hs683), human prostate cancer (PC3) and Brain primary metastases (Kaka).

Since adenocarcinomic human alveolar basal epithelial cells (A549) and glioblastoma cell (U373) are known to be more resistant to apoptosis stimuli than human prostate cancer (PC3) [25], the cytotoxic effect of SbDF against U373 (IC_{50} = 7µg/ml), A549 (IC_{50} = 9µg/ml) and PC3 (IC_{50} = 10µg/ml) could be a non-apoptosis dependent cytotoxic effect.

A series of ethnopharmacological surveys have indicated that more than 1,200 plants are used worldwide for their alleged hypoglycaemia activity while more than 3000 plant species are used against cancer [26-28]. Anticancer activity of Combretum genus are well documented in the literature. *Combretum* species are widely used in traditional medicine against a series of diseases such as cancer and diabetes [21-24]. *C. quadrangulare* leaf extract significantly induced cytotoxicity, apoptosis, cell cycle arrest and anti-migration of lung cancer cell lines [29, 30]. *C. fructinosum* induced autophagic cell death, with cytoplasmic vacuolization and formation of autophagosomes in HCT-116 colon carcinoma human cells [31].

Table 1: Cytotoxicity evaluation

Cancer cells lines	IC_{50} [µg/ml] Estimation		
	Extracts		
	SbMF	SbEAF	SbDF
A549	85	83	9
U373	> 100	83	7
Hs683	> 100	87	8.5
PC3	85	67	10
KaKa	> 100	60	8.3

Bio-assay guided fractionation

In bioassay, the cancer cell line A549 was used as test organism. Fractions from chromatographic columns below 50 mg have not been tested. Ten primary fractions (SbDF-1-SbDF-10) were obtained from silica gel column chromatography of SbDF (Figure 2), Among these, SbDF2

and SbDF3 were the most active with an IC_{50} of 6.3µg/ml and 5.8µg/ml, respectively. A combined fraction of SbDF2 and SbDF3 denoted as SbDF3a was fractionated into 7 subfractions through CC on SiO2. Of these, SbDF3a5 was the most cytotoxic with an IC_{50} = 4.8µg/ml. The fractionation of this sample led to SbDF3a52 with an IC_{50} of 2.8µg/ml. From this active subfraction, compounds 1-3 were isolated through repetitive CC on Silicagel and preparative TLC. Their structures were elucidated by NMR spectroscopic method.

The 1H NMR spectrum of compound 1 (400 MHz CDCl3, Table-2) clearly reveals a singlet at δ 6.57 integrating for one proton, which can be attributed to the flavone proton (C-3H). The 13C NMR spectrum (100 MHz CDCl3, Table-2) shows two signals at δ 55.50 and 55.75 indicating the presence of two methoxyls groups a carbonyl carbon at δ 182.44. Based on the concordance with the literature data compound 1 was identified as 5- hydroxy-7, 4'-dimethoxy flavone [32, 33].

The NMR spectrum (1H NMR, 13C NMR) of compound 2 (Table-3) displayed signals for typical pentacyclic triterpenoid methyl groups, with 13C absorptions at δ 19.32, 150.88 and 109.41 which are characteristic of the isopropenyl group of triterpenes of the lupene type. Based on the concordance with the literature data compound 2 was identified as lupenone [34].

The 1H NMR spectrum of compound 3 (400 MHz CDCl3, Table-3) revealed the presence of seven methyl singlets and olefinic function while the 13C NMR spectrum showed thirty carbons. Based on the concordance with the literature data compound 3 was identified as lupeol [34].

Thus, the compound is 5- hydroxy-7, 4'-dimethoxy flavone and was the first time reported from *C. glutinosum. Combretum. paniculatum* and *C. caffrum* were reported to exhibit a cytotoxicity against cancer cell line [35]. Martini *et al.* in 2004 reported that 7-4'-dimethoxy-5-hydroxy flavone isolated from *Combretum erythrophyllum* is potentially toxic for human cell [36]. According to Singh *et al*;, the anticancer and AGEs formation inhibitory activities could be related to the hydroxyl at C-5 on ring-A [37].

The antiproliferative effect against cancer cell line of some *Combretum* species have been described. This is the case of *C. leprosum* from which, the cytotoxicity of a pentacyclic triterpene against MCF-7 Breast cancer cell line with an IC_{50} of 0.30 µg/mL at 120 h has been indicated; Stilbene compounds such as Combretastatins isolated from *C. caffrum* and their analogues are known to act as anti-angiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis [35, 38, 39]. Combretastatins, a group of stilbenes with anti-tubulin and hence antimitotic activity have been isolated from the stem bark of *C. caffrum* from South African. Combrequinone A, B and C isolated from *C. yunnanense* were found cytotoxic against five cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) particulary against HL-60 acute leukemia cells, with IC_{50} values of 4.63, 4.07, and 1.26 µM [40]. Well *et al.*, reported that phytochemicals or a plant extract of leaves the *C. micranthum* can suppress insulin resistance, and lower the glucose production from gluconeogenesis [17]. An lyophilised ethanol extract of *C. micranthum* leaves is reported to be rich phytochemical constituents such as tannins, flavonoids and other components, constituents known to possess various beneficial pharmacological properties such as potential antioxidant, antimutagenic, anticarcinogenic, hypolipidemic and cardioproprotective activities [41].

Pheophorbide and his methyl ester isolated from *C. paniculatum* were found cytotoxic against MCF-7 and HeLa cancer cell line, and induce cycle arrest at the G0/G1 phase in the HeLa cell [42]. Like these cytotoxic and antitumor Combretum species, it is interesting to note that the most cytotoxic effect of *C. glutinosum* was also observed with the apolar extract (SbDF). Traoré *et al.*, reported that the chloroformic extract (IC_{50} = 20.5 µg/mL) of C. glutinosum was more cytotoxic than methanolic extract (IC_{50} > 64 µg/mL) against human fetal lung fibroblast (MRC-5) cells [43] suggesting *C. glutinosum* apolar extract was more cytotoxic against cancer cell line than normal cell line.

A phytochemical study of the genus Combretum demonstrated many classes of components including triterpenes, flavonoids, nonprotein amino acids and lignans [31]. Although widespread in plant species, this is the first finding of Lupenone and lupeol in *C. glutinosum*. These compounds have been also reported in *C. griffithii* [44] *C. mellifluum* [45]. Pharmacological screening of these two lupane type triterpenoids revealed various pharmacological activities including anti-inflammatory, anti-protozoal, anti-microbial anti-virus, anti-diabetes, and anti-cancer. About their anti-tumor potency, they were reported to have antiprostate cancer, antipancreatic cancer, antihead and neck squamous cell carcinoma, antimelanoma; when their IC_{50} values were compared, lupenone (25,4µM) was more active than lupeol (38µM) [46, 47]. On the other hand, lupenone and lupeol helped to promote the inhibition of protein tyrosine phosphatase 1B (PTP1B) which significantly decreased insulin activity in diabetes and treated obesity. Lupenone could decrease fasting blood glucose and hemoglobin A1c in blood of high-fat diet fed-streptozotocin-induced type 2 diabetic rats [48] and inhibit the PTP1B which is an attractive target for the development of new drugs for type 2 diabetes and obesity [34]

Conclusions

The assessed cytotoxic and antidiabetic activities of *Combretum glutinosum* could be linked at least partially to the presence of flavonoids such as 7-4'-dimethoxy-5-hydroxy flavone, and lupan type triterpenoides such as lupeol, and lupenone. These results are in accordance with the Guinean traditional use of C. glutinosum in the management of type 2 diabetes. Further phytochemical and biological investigations such as ethnotherapeutical and clinical trials are in progress to evaluate the effectiveness and tolerance of the leaves of C. glutinosum.

Conflicts of Interest

"The author[s] declare[s] that there is no conflict of interest regarding the publication of this paper."

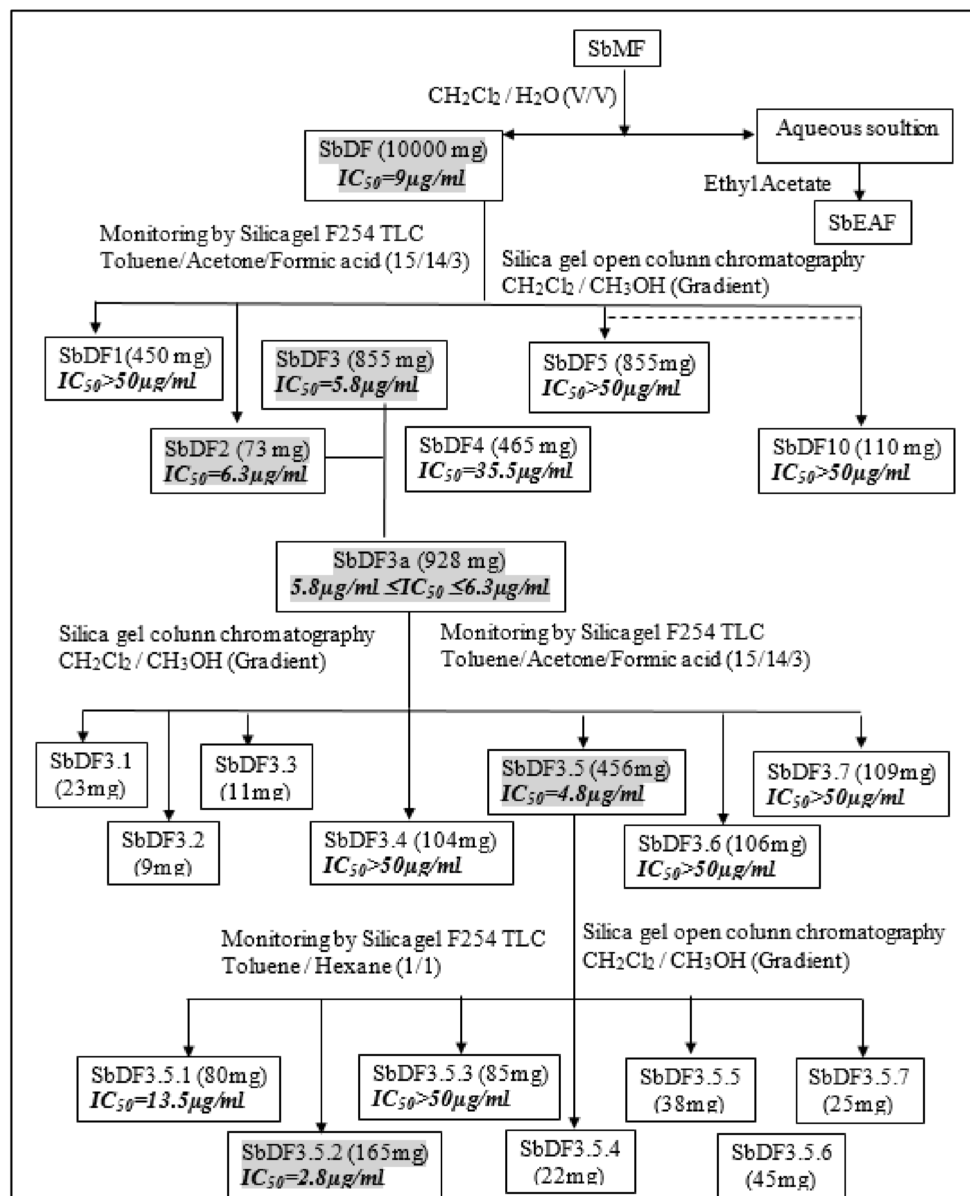

Fig 2: Bio-assay guided fractionation of the extract SbDF

Table 2: $^1H((CD_3)2S0$, 200 MHz) and ^{13}C (DMSO, 75 MHz) NMR spectral data of Compound1

Position	Compound 1		5- hydroxy-7, 4'-dimethoxy flavone ($CDCl_3$; 400 MHz) [32]	5- hydroxy-7, 4'-dimethoxy flavone (CDCl3, 100 MHz) [33]
	δH [*J* Hz]	δ C	δH [*J* Hz]	δ C
2		162.6		163.9
3	6,87 s	104.1	6,58 [s]	104.2
4		182.0		182.4
5		162.2		162.0
6	6,40 [d, J=4 Hz]	98.1	6,37 [d, J= 2,4Hz]	98.0
7		165.2		165.3
8	6,80 [d, J=4 Hz]	92.6	6,48 [d, J= 2,4Hz]	92.5
9		157.7		157.6
10		105.6		105.4
1'		123.6		123.4
2'	8,08 [d, J=8 Hz]	128.0	7,85 [d, J= 9Hz]	127.9
3'	7,10 [d, J=8 Hz]	114.5	7,02 [d, J= 9Hz]	114.4
4'		163.6		162.5
5'	7,10 [d, J=8 Hz]	114.5	7,02 [d, J= 9Hz]	114.4
6'	8,08 [d, J=8 Hz]	128.0	7,85 [d, J= 9Hz]	127.9
7-OCH_3	3,90 s	55.9	3.89 s	55.8
4'-OCH_3	3,90 s	55.6	3.88 s	55.5
12-OH	12,90 s		12,80 [s]	

Table 3: ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C ($CDCl_3$, 100 MHZ)- NMR spectral data of Compounds 2 and 3

Position	Compound 2		Lupenone [34]		Compound 3		Lupeol [34]	
	δH [*J* Hz]	δ C	δH [*J* Hz]	δ C	δH [*J* Hz]	δ C	δH [*J* Hz]	δ C
1		39.62		39.61		38.72		38.69
2		33.57		33.56		27.41		27.43
3		218.20		218.26	3.19 [1H, dd, J = 5.1, 11.2 Hz]	79.02	3.18 [1H, dd, *J* = 5.08, 11.32 Hz]	78.98
4		47.34		47.32		38.87		38.84
5		54.93		54.92		55.31		55.28
6		19.69		19.67		18.33		18.30
7		34.16		34.14		34.29		34.26
8		40.78		40.77		40.84		40.81
9		49.80		49.78		50.45		50.41
10		36.89		36.88		37.18		37.14
11		21.48		21.46		20.94		20.91
12		25.16		25.15		25.15		25.12
13		38.18		38.16		38.06		38.03
14		42.90		42.88		42.84		42.81
15		27.44		27.42		27.80		27.40
16		35.51		35.51		35.59		35.56
17		42.98		42.98		43.01		42.98
18		48.23		48.23		48.31		47.97
19		47.97		47.95	2.38 [1H, dd, J=5.6 ; 11.0Hz]	48.11	2.37 [1H,dd]	48.28
20		150.88		150.86		150.97		150.96
21		29.84		29.82		29.86		29.83
22		39.98		39.97		40.01		39.98
23		26.66		26.64	0.74 [3H,s]	28.00	0.78 [3H,s]	27.97
24	1.02 [3H,s]	21.05	1.02 [3H,s]	21.03	0.93 [3H,s]	15.38	0.94 [3H,s]	15.35
25	0.95 [3H,s]	15.96	0.93 [3H,s]	15.96	0.88 [3H,s]	16.13	0.83 [3H,s]	16.10
26	1.06 [6H,s]	15.80	1.07 [6H,s]	15.77	1.03 [3H,s]	15.99	1.03[3H,s]	15.96
27	0.98[3H,s]	14.49	0.95[3H,s]	14.47	0.93 [3H,s]	14.56	0.96 [3H,s]	14.53
28	0.81[3H,s]	18.02	0.79[3H,s]	18.00	0.74 [3H,s]	18.01	0.76 [3H,s]	17.98
29	4.71 [1H, d, *J* = 2.0 Hz] 4.59 [1H, d, *J* = 1.6 Hz]	109.41	4.69 [1H, d, *J* = 1.95 Hz] 4.57 [1H, d, *J* = 1.56 Hz]	109.39	4.70 [1H,s], 4.71 [1H,s]	109.33	4.68 [1H,s], 4,56 [1H,s]	109.31
30	1.68 [3H,s]	19.32	1.68 [3H,s]	19.30	1.68 [3H,s]	19.32	1.68 [3H,s]	19.29

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