International Journal of Modern Science and Technology, 2018;3(11):230-237.
ISSN: 2456-0235.
www.ijmst.co

Research Article

Phytochemical Analysis of *Mitracarpus villosus* and Comparative Toxicity of *Mitracarpus villosus* Ointment and Honey

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Abstract

Studies on compounds present in plant or animal matter helps in identifying compounds responsible for harmful and beneficial effect in plants and animal by-products. This study was designed to identify and quantify phytochemical compounds in *Mitracarpus villosus* methanolic leaf extract, and determine the toxicity of *M villosus* ointment and honey. The results show that *M. villosus* leaves contains phenolics, saponins, flavonoids, cardiac glycosides and tannins but no alkaloids. Quantitative phytochemical analysis showed varied percentage content of these compounds with saponins being highest (14.0%) and tannins lowest (1.41%). Acute oral toxicity was determined to be 15066 mg/kg and 7542 mg/kg as LD_{50} for honey and *M. villosus* ointment respectively, inferring that both honey and *M. villosus* ointment belong to the nontoxic class group of substances. Primary irritation indices were recorded as 0.16 and 0.33 for honey and ointment respectively. Therefore, honey and *M. villosus* ointment belong to the category of negligible irritants. These findings is indicative of the promising potentials of *M. villosus* ointment as a topical remedy for diseases and further confirms reasons for high demand of honey for various uses despite its alarming cost increase.

Keywords: *Mitracarpus villosus;* Ointment; Honey; toxicity; Phytochemical.

Introduction

Honey is a sweet natural product with flavour, and is produced by bees from plant nectars, plant secretions and excretions of plant-sucking insects. It is consumed for its high nutritive value and is a good source of natural macro- and micro-nutrients, consisting of a saturated solution of sugars, of which fructose and glucose are the main contributors, but also of a wide range of minor constituents, especially phenolic compounds [1, 2].

The composition of honey which influences its biological effects is rather variable and depends primarily on its floral source, seasonal and environmental factors [3]. Several studies have shown that the antioxidant potential of honey is strongly correlated with the concentration of phytochemicals: phenolics, flavonoids, saponins and tannins and are important chemical factors that provide the healing properties to honey [2]. Clearance of infections, rapid debridement of wounds, rapid suppression of inflammation, minimization of scarring, and stimulation of angiogenesis as well as tissue granulation and epithelium growth are factors responsible for the use of honey in wound dressing [4].

Mitracarpus villosus (sw) Dc, is a plant with the synonym *Mitracarpus scaber* zucc. ex schult belongs to the family *Rubiaceae*. The plant is commonly known by the names Goga-masu, sirawo ile, obuobwa, and Antyo kpoughloo, in Hausa; Yoruba; Igbo; and Tiv respectively. It is an annual, erect that can grow up to 60 cm tall. The stem is angled, hairy and sparsely branched. It is woody at the base, segmented into nodes with each internode bearing a pair of leaves. The fresh leaves are green in colour with the characteristic mild odour, bitter and peppery taste. The leaf is simple in composition, opposite/decussate in arrangement, lanceolate in shape, entire in margin with a cuneate base and an acute apex. Venation is parallel, without petiole i.e. sessile and with an internode length of 5.2-7.2 cm. Veins are more prominent on lower surface with nearly glabrous upper and lower surfaces with hairs on mid-rib region**.** The plant is a common

Received: 03.10.2018; Received after Revision: 20.10.2018; Accepted: 24.10.2018; Published: 14.11.2018

weed in upland areas from the forest to the savannah zones. It is widespread in tropical Asia and Africa [5, 6].

Studies have shown that aqueous, ethanolic, methanolic and a combination of solvent extracts of *M. villosus* contain Alkaloids, saponins tannins, flavonoids, phenolics, glycosides, resins, steroids and terpenoids [5,7]. Though variations have been observed in the presence of these secondary metabolites, a variability occasioned by the solvent of extraction, and plant part used. However, consistency has been observed in the presence of saponins, tannins, flavonoids, and phenolics with no regards to solvent and plant part used in. Gallic acid, 4-methoxyacetophenone, 3,4,5-trimethoxy acetophenone, 3,4,5 trimethoxybenzoic acid, Kaempferol- 3-O-rutinoside, rutin, stigmasterol, 24-methylcholesta-5-en-3*β*-ol, and ursolic acid, hydroquinone diglycoside, harounoside, benz[g]isoquinoline-5,10-dione, oleanolic acids and psoralen have been isolated and identified from *M. villosus* [8, 9].

Secondary metabolites observed to be consistent in *M. villosus* extracts are known for their antioxidant, antibacteriacidal, antipyretic, wound healing activities and other disease remedies [10]. The presence and activities of these bioactive compounds in *M. villosus* is the reason for its wide use in traditional medicine in West Africa for treatment of headache, toothache, amenorrhea, dyspepsia, hepatic diseases and, leprosy, wounds, rheumatic places, skin infections cuts, ulcers, boils, and wounds [2].

Nature's providence to man for nutrition, therapeutic and other purpose is not restricted to honey and *M. villosus*. Several plants have been discovered for their beneficial and harmful effects to man. However much work still needs to be done in the areas of: identifying these plants and animal by-products beneficial and harmful to man, identification and quantification and isolation of the bioactive compounds responsible for these effects and proffering possible means of improving or limiting desired or none desired effects. This study considers identification and quantification of phytochemicals of *M. villosus* plant and the toxicity of the plant ointment and honey.

Materials and methods

Ointment preparation

The plant ointment was prepared according to the method described by [11]. 500 g of *M. villosus* leafs was washed with tap water to remove soil/dust and air dried for 15 days. The leaves was then ground into powder with a Linsan® blending machine (Lin 319) at 1000RPM for 10 min at 25°C.

Methanol extract was prepared by stirring 100 g of *M. villosus* in 500 cm^3 of methanol on a magnetic rotor (Jenway® 1000) at 100RPM for 12 h and then centrifuged at 25 g. The supernatant was collected and evaporated to dryness at 250 atm in a rotary evaporator (IKA® EW-28710-22). The extract was aliquoted in amber-coloured bottles and kept in desiccators at 250°C for further use.

To prepare (50% w/w) *M. villosus* ointment, 8 g of soft white paraffin was suspended in mortar suspended on a hot water bath and heated for 30 min to melt. Then 8 g of the extract was dissolved in 20 ml dimethyl sulphuroxide (DMSO) from JHD® Ltd and mixed thoroughly with the melted paraffin for 20 min

Qualitative and Quantitative Phytochemical Analysis

The quantitative and qualitative determination of phytochemicals in the methanolic leaf extracts of *M villosus* was done using the methods employed by [12, 13]. Ferric chloride was used to test for presences of phenolics: 0.5 cm^3 of extract was treated with few drops 5% ferric chloride and observed for formation of deep blue or black colour. Frothing test- 0.2 g of concentrate was taken in a test tube. 5.0 cm^3 of water was added and vigorously shaken. A persistent froth that last for at least 15 minutes indicates the presence of saponins. 5 cm^3 of the methanolic extract was mixed with 2 cm^3 of glacial acetic acid containing one drop of ferric chloride ($FeCl_3$) solution, followed by the addition of 1 cm^3 concentrated sulphuric acid. Brown ring was formed at the interface which indicated the presence of deoxysugar of cardenoloides. A violet ring appeared beneath the brown ring, while in the acetic acid layer, a greenish ring also formed just gradually throughout the layer. For flavonoids, 3 cm^3 of the extract was mixed with 4 cm^3 of 1%

potassium hydroxide in a test tube and the colour was observed. A dark yellow colour indicated the presence of flavonoids. In testing the presences of alkaloids, 2 cm^3 of extract was taken in a test tube and then 0.2 cm^3 dilute HCl was added, followed by 1 cm^3 of Meyer's reagent. A yellowish coloration indicates alkaloid's presence.

Flavonoids

The extract (5 cm^3) was transferred in to a small flask and then hydrolysed by heating on a water bath with 10% H_2SO_4 (10 cm^3) for 30 min. The original volume was reduced to half and the mixture was cooled on ice for 15 min where the flavonoids were precipitated. The cooled solution was then filtered and the residue was dissolved by pouring warm 95% ethanol (50 cm^3) and further made to 100 cm^3 with 95% ethanol. Aliquot (5 cm^3) was pipetted into a 25 cm^3 volumetric flask and diluted to volume with 50% ethanol. The absorbance of the resulting solution was measured spectrophotometricaly (Jenway 7305) at 370 nm against 50% ethanol blank. The flavonoid concentration was finally calculated using a reference curve of pure quercetin.

Tannins

Plant extract (5 cm^3) was transferred to a stoppered conical flask and 0.1N Iodine (25 cm^3) and 10% of 4% NaOH was added. This was mixed and kept in the dark for 15 min. The mixture was diluted with water and acidified with 4% H_2SO_4 (10 cm^3). The mixture was then titrated with 0.1N Sodium thiosulphate solution using starch solution as indicator. The volume (cm^3) of 0.1N Iodine used corresponds to the total tannins. Additionally, a blank experiment was carried out using distilled water.

Phenolics

Phenolics were determined spectrophotometricaly (Jenway 7305) 0.5 g of extract was boiled with 50 cm^3 of ether for 15 min, and 5 cm^3 of the boiled sample pitted into 50 cm^3 flask and 10 cm^3 of distilled water added. After addition of the distilled water, 2 cm^3 of NH_4OH solution and 5 cm^3 of concentrated pentanol were added to the mixture. Samples were then made up to mark and left for 30 min to react for colour development and measure at 505 nm wavelength

Saponins

The plant extract (50 cm^3) was placed in a 500 cm^3 flask, 50% alcohol (300 cm^3) was added and boiled under reflux for 30 min and filtered while hot through a coarse filter paper. Charcoal (2 g) was added to the filtrate, boiled and filtered again while hot. The filtrate was cooled and an equal volume of acetone was added to completely precipitate the saponin. The precipitated saponin was collected by decantation and dissolved in small amount of boiling 95% alcohol and filtered while hot. The filtrate was cooled to room temperature to separate the saponins in a relatively pure form. The clean supernatant fluid was decanted and the saponins suspended in alcohol (20 cm^3) and filtered. The filter paper was then transferred to a desiccator containing anhydrous calcium chloride and left to dry and weighed.

Experimental animals

Wistar rats weighing 160-250 g were allowed to acclimatize for 7days prior to the initiation of the experiment in cages and under laboratory conditions (temperature 22 ± 2 ^{0}C, relative humidity 60-70%, and 12-12 h light-dark cycle). Animals were fed with balanced diet purchased from UAC foods Nigeria limited and water *ad libitum*.

Experimental design

Oral acute toxicity for ointment and honey was determined by oral administration single doses of 2000 mg/kg, 4000 mg/kg, 5000 mg/kg, 10000 mg/kg, 15000 mg/kg and 2000 mg/kg, 4000 mg/kg, 10000 mg/kg, 15000 mg/kg, 20000 mg/kg respectively to a total of 30 rats (n= 3 per group), and observed for a period of 24 hours and the next 14 days. The dermal toxicity was done for 21days with 18rats which were grouped into 3 groups (n=6) where group 1= control (none treated), group 2= honey oral LD_{50} treated, and group 3= ointment oral LD_{50} treated

Determination of acute and dermal toxicity of honey and Mitracarlus villosus ointment

Acute toxicity was determined by the graphical method of Miller and Tainter as described in [14]. The animals were observed for toxicity symptoms and the percentage mortality was converted into probit referring to the probit table. The values thus obtained were plotted against log dose. The LD_{50} value and its standard

error were determined from the straight line graph.

To test for skin irritation, the furs were removed by closely clipping to an area of about 2 cm^2 on the dorsal surface of the trunk of the animals, about 24 h before the analysis. Care was taken to prevent scraping the skin, and only healthy animals with intact skin were used. The materials were applied to the shaved skin area of animals and the area covered with a gauze patch that was held in place with non-irritating tape. The patch was loosely held in contact with the skin by means of an appropriate semi-occlusive dressing. Restrainer (neck collar) was used to avoid the ingestion of the test substance from the application site. The residual substance was washed and the skin reaction of established therapeutic dose was examined and scored using the Draize dermal irritation scoring system (DDISS) at the first patch removal for 24 h and 72 h as contained in the OECD 402 Guideline on Acute Dermal Irritation/Corrosion [15].

Results and discussion

Qualitative and quantitative phytochemical analysis of M. villosus methanolic extract

The results of phytochemical analysis of the methanolic extract are presented in table 1. Qualitative analysis indicates that, there is presence of the phenolics, flavonoids, cardiac glycosides, saponnins, and tannins. Further quantitative assessment of the extract showed various quantities of phenolics, saponnins, flavonoids and tannins (12.00%, 16.00%, 2.65% and 1.41%) respectively. Saponins recorded the highest concentrations as opposed to the least concentration of tannins. These findings agree with the submissions of [5, 6].

Contrary to this finding, the absence of phenolics, cardiac glycosides, flavonoids and tannins and the presence of alkaloids have been reported [16]. These variations may be as a result of geographical variation. Varied effects of sun shine, and altitude on the composition of phytochemicals in *Moringa oleifera* has been reported with reference to these factors [17]. Whereas sunshine favoured the synthesis and increased activity of some compound in the sampled plants, low synthesis and activity was reported in other compounds [17].

Table 1. Qualitative and quantitative phytochemical analysis of methanolic extract of *M. villosus* leaves

S/N	Phytochemical	Qualitative Analysis	**Quantitative analysis** (%)
1	Phenolics	+++	12.00
2	Saponins	++	14.00
3	Flavonoids	++	2.65
4	Cardiac glycosides	++	ND
5	Tannins	++	1.41
6	Alkaloids	-	ND

***Key*=** ND= not determined, - = absent, + = Present

Acute oral toxicity of M. villosus ointment in Albino rats

Results for oral administration of *M. villosus* ointment at different doses in rats are presented in Table 2. They show that the rats administered *M. villosus* ointment had difficulty in respiration, reluctance to move, inability to remain upright, poor vision, slight diarrhoea, partial paralysis of lower limbs, and paralysis of lower limbs as observed symptoms. Least percentage death of 33.3% was recorded at 5000 mg/kg while 100% death at 15000 mg/kg. In Figure 1 the log-dose probit curve of albino rats exposed to *M. villosus* ointment is presented. The LD_{50} of *M. villosus* ointment in rats was determined to be 7542.9 mg/kg (3,943.97-11,141.8; 95% confidence intervals) after oral gavage of *M. villosus* ointment.

The LD_{50} of *M. villosus* ointment was determined to be above 5000 mg/kg of body weight for a single dose within 24 h. This finding places the ointment under the category of non-toxic substances based on the OECD classification [18]. The result obtained in this study (7542.9 mg/kg) is far greater than that of [10] (885 mg/kg) in mice. This variation may be the result of interactions of bioactive compounds of the plant with other compounds in the ointment mixture. Some of these types of interactions may not be favourable in that, they increase the toxicity of the mixture. Conversely, other interactions are beneficial because they lower the toxicity of the mixture as in the case of *M. villosus* ointment [19]. The findings of this study agrees with the report that *M. villosus* has an LD_{50} >5000 mg/kg of body weight [20].

Acute oral toxicity of honey in Albino rats

In Table 3, the results for oral administration of honey in rats are presented. They show that the rats administered honey had symptoms such as reduced food and water consumption, corner sitting, difficulty in respiration, reluctance to move, inability to remain upright. A least percentage death of 33.3% was observed at 15000 mg/kg and 100% death at 20000 mg/kg. Figure 2 show the log-dose probit curve of albino rats exposed to honey. The LD_{50} of honey in rats was determined to be 15,066.1 mg/kg (12, 2735.8-17,858.42; 95% confidence intervals) after oral gavage.

Table 2. Probit values of albino rats exposed to *M. villosus* ointment

Groups	Dose (mg/kg)	Log-Dose	% Dead	Corrected value	Probit value	Observed Symptoms
I	2000	3.30103	0	-	-	RFWC, CS, fatigue
II	4000	3.60206	0	8.33	3.59	CS, Poor vision, IWI, SD,
III	5000	3.69897	33.3	33.3	4.56	RFWC, Emaciation, SD
IV	10000	4	66.7	6.7	5.41	RFWC, Emaciation, CS, PPLL
V	15000	4.176091	100	91.67	6.3	RFC, IWI, Emaciation, CS

Key=RFWC -Reduced food and water consumption, RFC- Reduced food consumption, IWI- increased water intake, CS- Corner sitting, DR - difficulty in respiration, RM - reluctance to move, IRU - Inability to remain upright, PV- poor vision, SD- slight diarrhoea, PPLL- partial paralysis of lower limbs, PLL- paralysis of lower limbs.

Table 3. Probit values of Albino rats exposed to honey

Groups	Dose (mg/kg)	log Dose	% Dead	Corrected Value	Probit Value	Observed Symptoms
I	2000	3.30103	0	-	-	
II	5000	3.69897	0	-	-	
III	10000	4	0	8.33	3.59	RFWC
IV	15000	4.176091	33.3	33.3	4.56	RFWC, CS, DR, RM, IRU Emaciation.
V	20000	4.30103	100	91.67	6.3	RFWC, CS, DR, RM, IRU Emaciation.

Key=RFWC -Reduced food and water consumption, RFC- Reduced food consumption, CS- Corner sitting, DR - difficulty in respiration, RM - reluctance to move, IRU - Inability to remain upright.

The LD_{50} for honey in Albino rats was determined to be >5000 mg/kg of body weight for single dose oral administration within 24 h. Based on OECD classification, honey belongs to non-toxic class of substances [18]. The LD_{50} of >5000 mg/kg and absence of clinical symptoms at a dose of ≤5000 mg/kg are in agreement with the report of [21] for galem honey. An LD_{50} in rats of >5000 mg/kg for an herbal formulation which contains about 50% of honey have also been reported. This put honey in the position of substances that can work in synergy and have a possible toxicity lowering activity. Despite the wide acceptability of honey as a safe liquid for consumption it may contain toxins: hyenanchin, euphorbic acid, acetylandromedol and ericolin.

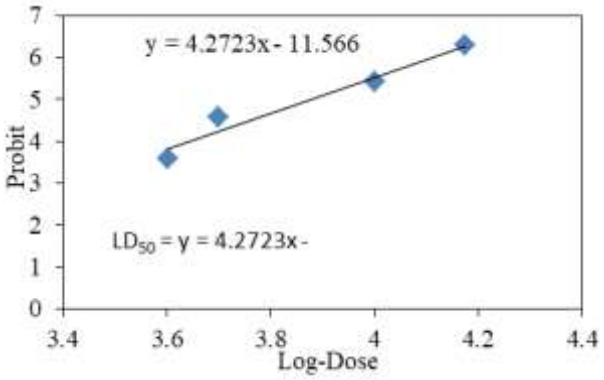

Figure 1. Log-Dose probit curve of albino rats exposed to *M. villosus* ointment

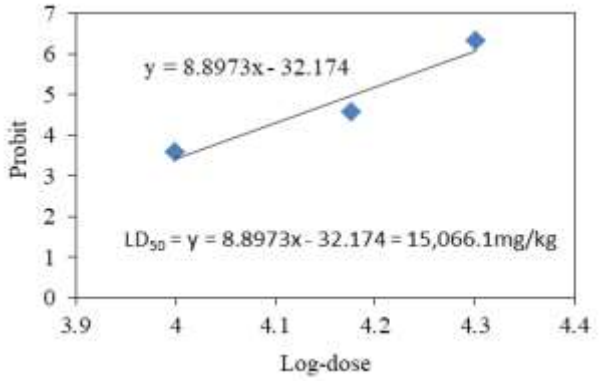

Figure 2. Log-Dose probit curve of Albino rats exposed to honey

These toxins are heat resistant and can cause poisoning case if quantities as low as 5-10 g are present in honey [22]. Respiratory symptoms observed in the rats were due to the presence of saponins in the plant as they have been implicated to cause paralysis to the respiratory system that may be lethal [20]. Saponins have also been reported to cause diarrhoea [23]. Honey is also reported to contain these secondary metabolites [1, 2].

Table 4. Dermal toxicity of *M. Villosus* ointment and honey in albino rats

Groups	TOA h	Number of Rats 1 Er	1 Ed	2 Er	2 Ed	3 Er	3 Ed	4 Er	4 Ed	5 Er	5 Ed	6 Er	6 Ed	Sum Er+Ed	Dermal toxicity
Control	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00
	72	0	0	0	0	0	0	0	0	0	0	0	0		
HT	24	0	0	0	0	0	0	0	1	0	0	0	0	2	0.16
	72	0	0	1	0	0	0	0	0	0	0	0	0		
OT	24	0	0	0	0	0	0	1	0	0	0	1	0	4	0.33
	72	0	0	0	0	0	1	1	0	0	0	0	0		

Key: TOA=time of assessment, Er=erythema, Ed=edema, OT = Ointment Treatment, HT = Honey treatment

Dermal toxicity= $\frac{\sum(Er+Ed)}{No\ of\ test\ sites\ per\ animal\ x\ 2}$; where, 2 = scoring time intervals (24 and 72 h)

Dermal toxicity of LD_{50} of acute oral toxicity of M. villosus ointment and honey in albino rats

In the present study, the irritation potential of *M. villosus* ointment after a single dose application was investigated. Based on the results obtained, the ointment is considered a negligible irritant on the skin of rats for single exposure. An irritation reaction is described as a localised inflammation that follows to dermal penetration of irritant agents. This is due to the removal of surface lipids or water soluble substances and denaturation of scleroproteins of the horny layer leading to progressive skin irritation and damage [25]. Dermal toxicity of honey after single dose application was investigated. The results show that honey may be considered negligible irritant on the skin for single exposure. Honey has been reported to be a slight irritant to skin and eyes due to interactions of biochemical of honey on the skin of test models [26].

Conclusions

From the findings in the present study, it was concluded that *Mitracarpus villosus* leaf extract contains phenolics, flavonoids cardiac glycosides saponins and tannins but no alkaloids and that *Mitracarpus villosus* ointment like honey is a non-toxic substance whether administered orally or topical. Therefore recommendation is made that the plant ointment be studied for topical in wound healing and treatment of other skin related diseases.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Mr. Itolo Ogli of Biochemistry Department, College of Science, University Agriculture Makurdi and Mr. Vincent Upev of Biochemistry Department, College of Veterinary Medicine, University Agriculture Makurdi for assistance with reagents for phytochemical analysis.

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