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Full Length Research Paper

DPPH radical scavenging activity of extracts from *Rhamnus prinoides*

Manoharan Karuppiyah Pillai^{1*}, Lehlohonolo Isaac Santi¹ and Sibusisiwe Magama²

¹Department of Chemistry and Chemical Technology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, South Africa.

²Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, South Africa.

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Hexane, chloroform, ethyl acetate and methanolic extracts from leaves and stem-bark of *Rhamnus prinoides* were evaluated for their antioxidant activity by DPPH radical scavenging assay. The leaves extracts showed scavenging activity ranging from 03.33 ± 0.89 to $55.03 \pm 3.40 \mu\text{g mL}^{-1}$ while the stem-bark extracts showed relatively strong scavenging activity ranging from 03.65 ± 1.02 to $59.55 \pm 2.27 \mu\text{g mL}^{-1}$. The IC_{50} values of *R. prinoides* hexane leaves extract (RPHELs), *R. prinoides* chloroform leaves extract (RPCHLS), *R. prinoides* ethyl acetate leaves extract (RPEALS), *R. prinoides* methanolic leaves extract (RPMELS), *R. prinoides* hexane stem-bark extract (RPHEsB), *R. prinoides* chloroform stem-bark extract (RPCHsB), *R. prinoides* ethyl acetate stem-bark extract (RPEASB) and *R. prinoides* methanolic stem-bark extract (RPMESB) were found to be >3000 , >3000 , >3000 , 950.42 , ~ 1500 , 710.50 , ~ 1000 and $902.78 \mu\text{g mL}^{-1}$, respectively. The positive control ascorbic acid showed an IC_{50} value of $<200 \mu\text{g mL}^{-1}$. From this study, we concluded that the extracts from *R. prinoides* showed promising antioxidant activity. *R. prinoides* finds therapeutic applications in the traditional medicine. Further research is required to commercialize products from this plant.

Key words: Antioxidant, ascorbic acid, *Rhamnus prinoides*, radical scavenging assay, methanolic extract, chloroform extract, hexane extract, ethyl acetate extract.

INTRODUCTION

Rhamnus prinoides belongs to the Rhamnaceae family (Dale, 2000; Dlamini and Turner, 2002). *R. prinoides* is also known by other names such as African Dogwood, Glossy-leaf and miffi. *R. prinoides* is widely distributed in East and South African countries (Alemu et al., 2007; Abegaz et al., 1999) which include Ethiopia, Botswana, Eritrea, Lesotho, Namibia, South Africa, Swaziland,

Uganda and Kenya (Ashine, 2015). *R. prinoides* grows up to 4.5-m height and found in evergreen forests, in the wild and along water streams. Although, *R. prinoides* is a slow growing plant in low rainfall areas, it can grow 1 m per annum in wet areas (Ferede et al., 2018). The leaves begin with pale green and turn to dark and shiny on maturation. The roundish red berries attract bees and

*Corresponding author. E-mail: kmharan@rediffmail.com. Tel: +266 5221 3499.

domestic fowl. *R. prinoides* casts a very deep shade such that it will not allow other plants to grow around it. *R. prinoides* flowers towards the end of the year and fruiting occurs at the beginning of the year. *R. prinoides* finds therapeutic applications in the traditional medicine. The decoction of roots has been used to treat pulmonary tuberculosis, pneumonia, bladder and kidney problems (Maliehe, 1997; Van Wyk and Moteetee, 2011). The bark has been used to induce vomiting. An extract of the root together with the bark of *Erythrina tomentosa* has been used to relieve colic. The leaves have been applied as a liniment to simple sprains. *R. prinoides* has been used to provide a special aroma and flavor (Shale and Gashe, 1991; Abegaz et al., 1999). *R. prinoides* has also been used in the beer industry as a hopping agent. Geshoidin, a naphthalenic glycoside, present in the stem-bark is responsible for providing bitterness in alcoholic beverages (Nindi et al., 1999). The antioxidant activity of 97% ethanolic extracts from leaves of *R. prinoides* has been reported previously (Ashine, 2015). Methanolic and aqueous extracts from roots of *R. prinoides* have also been evaluated for their DPPH radical scavenging activity (Kimondo et al., 2019). However, to the best of our knowledge, the scavenging activity of hexane, chloroform, ethyl acetate and methanolic extracts of the leaves and stem-bark of *R. prinoides* has not been reported previously, particularly the plant species gathered from the Kingdom of Lesotho. The aim of the present study was to evaluate the antioxidant activity of these extracts by DPPH radical scavenging assay and to determine their IC₅₀ values. The results obtained are communicated in this article.

MATERIALS AND METHODS

Plant materials

The leaves and stem-bark of *R. prinoides* were collected from the foothills of Popa and Popanyane Mountains at Mokhokhong village, Roma, Maseru district, the Kingdom of Lesotho, Southern Africa in January 2019. A voucher specimen viz. Santi/RPLS/2019 for leaves and Santi/RPSB/2019 for stem-bark were kept separately in the Organic Research Laboratory, Department of Chemistry and Chemical Technology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, Maseru district, Kingdom of Lesotho, Southern Africa.

Processing of materials

The leaves were allowed to air dry at room temperature for two weeks and then ground into powder using a commercial blender (Waring Blender, Blender 80119, Model HGB2WT93, 240V AC, 50-80 Hz, 3.6AMPs, Laboratory and Analytical Supplies). The chopped stem-barks were allowed to air dry at room temperature for two weeks and ground into powder using the blender.

Preparation of plant extracts

The powdered leaves (300.043 g) of *R. prinoides* were extracted

with methanol under cold conditions for 3 days. The solution was filtered using a filter paper (Boeco, Germany). The solvent was removed by *vacuo* and the same procedure was repeated once again. Finally, the plant material was extracted with hot methanol. 40.1858 g of combined methanol extract was obtained after removal of solvent. The same extraction procedure was followed to get hexane (3.2274 g), chloroform (10.6285 g) and ethyl acetate (11.4763 g) extracts from 300.254, 300.131 and 299.921 g of powdered leaves, respectively. The powdered stem-bark (299.530 g) of *R. prinoides* was extracted first with methanol at room temperature for 3 days followed by a reflux condition for 6 h. 32.2047 g of combined crude methanol extract was obtained after removal of solvent. The same extraction procedures were followed to get hexane (2.5895 g), chloroform (5.4327 g) and ethyl acetate (8.1493 g) extracts from 300.014, 300.157, and 300.422 g of powdered stem-bark, respectively.

Chemicals and solvents used

Ascorbic acid, DPPH, hexane (AR grade, 99.5%), chloroform (AR grade, 99.5%), ethyl acetate (AR grade, 99.5%) and methanol (AR grade, 99.5%) were all purchased from Sigma-Aldrich.

Antioxidant activity

The antioxidant activity of the extracts was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described in literature (Kim et al., 2002). Briefly, stock solution of the methanolic extract was prepared at a concentration of 3.0 mg of extract in 1 mL of 50% methanol (v/v). Serial dilutions were made from this stock solution to obtain solutions with concentrations of 3000, 2000, 1500, 1000, 800, 500 and 200 µg mL⁻¹. Solutions without extract concentration served as negative control. A solution of 3.94 mg of DPPH in 100 mL of methanol served as oxidant which was prepared just before use and stored in dark to minimize degradation. 0.1 mL sample of plant extract solution was mixed with 1.0 mL of 0.1 mM DPPH solution and 0.45 mL of 50 mM Tris-HCL buffer (pH 7.40). Similarly, stock solutions of hexane, chloroform and ethyl acetate extracts were prepared at a concentration of 3.0 mg of extract in 1 mL of 50% methanol (v/v). Further dilutions were made from these stock solutions to obtain solutions with concentrations of 3000, 2000, 1500, 1000, 800, 500, 200 and 0 µg mL⁻¹. 0.1 mL each of extract was mixed separately with 1.0 mL of 0.1 mM DPPH solution and 0.45 mL of 50 mM Tris-HCL buffer (pH 7.40). A stock solution of ascorbic acid (0.3 g) in 50% methanol (v/v) was prepared and serial dilutions were made as previously and served as positive control. 0.1 mL was mixed with 1.0 mL of 0.1 mM DPPH solution and 0.45 mL of 50 mM Tris-HCL buffer (pH 7.40). The mixtures were incubated for 30 min and their optical density was measured at 517 nm. Percentage inhibition of DPPH free radical was calculated using the equation:

$$\text{DPPH Scavenged (\%)} = [(A_{\text{cont}} - A_{\text{test}})/A_{\text{cont}}] \times 100$$

where A_{test} = Absorbance in the presence of extract or positive control and A_{cont} = Absorbance of negative control.

The IC₅₀ value is defined as the concentration (in µg mL⁻¹) of extract that inhibits the formation of DPPH radical by 50% (Moyo et al., 2013; Ndhala et al., 2013). A lower value of IC₅₀ represents higher antioxidant activity. The IC₅₀ values were calculated from graphs by plotting extract concentrations vs. percentage inhibition of DPPH radical using Microsoft Excel. Each experiment was carried out in triplicate and the averages of the three values were used to calculate IC₅₀ values. Standard deviation was calculated for each concentration from the three values of the experiment.

Statistical analysis

Results were expressed as means of three determinations. One-way analysis of variance (ANOVA) was used to compare means at the significance level $p < 0.05$. All analysis was performed by Microsoft Excel software.

RESULTS AND DISCUSSION

Table 1 summarizes the DPPH radical scavenging activity of hexane, chloroform, ethyl acetate and methanolic extracts of the leaves and stem-bark of *R. prinoides*. *R. prinoides* hexane leaves extract (RPHELS) showed 3.33 ± 0.89 , 3.80 ± 2.60 , 8.61 ± 1.39 , 10.12 ± 0.84 , 13.39 ± 2.94 , 20.10 ± 3.23 and $34.56 \pm 6.51\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. The positive control, ascorbic acid, showed 53.01 ± 3.98 , 53.46 ± 0.14 , 53.51 ± 0.77 , 53.82 ± 0.54 , 54.12 ± 1.64 , 54.34 ± 0.92 and $56.45 \pm 5.45\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. These results revealed that RPHELS showed very weak radical scavenging activity relative to positive control at all concentrations. *R. prinoides* chloroform leaves extracts (RPCHLS) showed 23.01 ± 3.44 , 27.15 ± 5.18 , 30.35 ± 1.02 , 31.38 ± 0.11 , 47.37 ± 4.14 , 48.38 ± 4.15 and $48.49 \pm 3.17\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. This result showed that RPCHLS has lower activity than positive control at all concentrations. *R. prinoides* ethyl acetate leaves extract (RPEALS) showed 17.71 ± 1.02 , 17.96 ± 0.82 , 19.48 ± 1.50 , 28.51 ± 0.62 , 36.05 ± 6.26 , 38.94 ± 5.75 and $42.33 \pm 5.88\%$ of scavenging activity at 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. This result revealed that RPEALS exhibited weak activity at low concentrations relative to positive control. However, at high concentration of $3000 \mu\text{g/mL}$, it showed higher scavenging activity of $42.33 \pm 5.88\%$. *R. prinoides* methanolic leaves extract (RPMELS) showed 21.01 ± 3.80 , 33.74 ± 1.42 , 41.50 ± 2.73 , 50.93 ± 5.27 , 52.93 ± 2.88 , 54.34 ± 2.70 and $55.03 \pm 3.40\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. This result revealed that RPMELS exhibited weak activity at low concentrations relative to positive control. However, RPMELS has comparable activity as that of positive control at high concentrations.

R. prinoides hexane stem-bark extract (RPHEBS) showed 12.39 ± 3.19 , 18.93 ± 1.04 , 30.47 ± 0.82 , 42.15 ± 4.06 , 50.24 ± 1.50 , 51.52 ± 2.47 and $52.15 \pm 1.06\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. This result revealed that RPHEBS exhibited weak activity at low concentrations relative to positive control. However, at higher concentrations such as 1500, 2000 and $3000 \mu\text{g/mL}$, RPHEBS exhibited comparable activity as that of positive control. *R. prinoides* chloroform stem-bark extract

(RPCHSB) showed 42.37 ± 5.65 , 43.30 ± 2.98 , 52.23 ± 2.46 , 53.64 ± 4.42 , 54.06 ± 1.41 , 54.41 ± 2.17 and $55.22 \pm 2.48\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. Thus, RPCHSB exhibited comparable activity as that of positive control at all concentrations except at concentrations of 200 and $500 \mu\text{g mL}^{-1}$. RPCHSB showed 42.37 ± 5.65 and $43.30 \pm 2.98\%$ of scavenging at concentrations 200 and $500 \mu\text{g/mL}$, respectively, while the positive control showed 53.01 ± 3.98 and $53.46 \pm 0.14\%$ of scavenging activity at concentrations 200 and $500 \mu\text{g mL}^{-1}$, respectively. *R. prinoides* ethyl acetate stem-bark extract (RPEASB) showed 3.65 ± 1.02 , 40.04 ± 1.50 , 47.09 ± 4.36 , 50.31 ± 7.80 , 51.61 ± 2.27 , 54.23 ± 1.83 and $57.43 \pm 3.28\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g/mL}$, respectively. Thus, RPEASB exhibited very weak activity relative to positive control at low concentration of $200 \mu\text{g mL}^{-1}$. *R. prinoides* methanolic stem-bark extract (RPMESB) showed 30.09 ± 5.26 , 41.69 ± 2.27 , 45.80 ± 2.54 , 52.78 ± 6.43 , 55.37 ± 3.90 , 56.19 ± 3.58 and $59.55 \pm 2.27\%$ of scavenging activity at concentrations 200, 500, 800, 1000, 1500, 2000 and $3000 \mu\text{g mL}^{-1}$, respectively. This result revealed that RPMESB exhibited weak activity at low concentrations relative to positive control. However, at high concentrations such as 2000 and $3000 \mu\text{g/mL}$, it showed higher scavenging activity of 56.19 ± 3.58 and $59.55 \pm 2.27\%$, respectively. Among the extracts (RPHELS, RPCHLS, RPEALS, RPMELS, RPHEBS, RPCHSB, RPEASB and RPMESB) from *R. prinoides*, RPMESB showed the highest scavenging activity (Table 1). For comparison and clarity, the percentage of scavenging activity of these extracts at various concentrations are as shown in Figures 1 and 2. The IC_{50} values of hexane, chloroform, ethyl acetate and methanolic extracts of the leaves and stem-bark of *R. prinoides* are shown in Table 2. RPHELS, RPCHLS, RPEALS, RPMELS, RPHEBS, RPCHSB, RPEASB and RPMESB exhibited IC_{50} values of >3000 , >3000 , >3000 , 950.42 , ~ 1500 , 710.50 – 1000 and $902.78 \mu\text{g mL}^{-1}$, respectively. RPCHSB is the most potent with IC_{50} value of $710.50 \mu\text{g mL}^{-1}$. The positive control ascorbic acid showed an IC_{50} value of $<200 \mu\text{g mL}^{-1}$.

The DPPH radical scavenging activity of 97% ethanolic extract from leaves of *R. prinoides* has previously been reported and its maximum radical scavenging was found to be 81.148% at a concentration of 24 mg mL^{-1} (Ashine, 2015) and the IC_{50} value was determined to be 5.2 mg mL^{-1} . The positive control, ascorbic acid, showed 93.77% scavenging activity with an IC_{50} value of 0.24 mg mL^{-1} in the same assay (Ashine, 2015). Therefore, when compared with the present study on the hexane, chloroform, ethyl acetate and methanolic extracts from leaves of *R. prinoides*, this 97% ethanolic extract from leaves of *R. prinoides* showed higher radical scavenging activity and lower IC_{50} value. This 97% ethanol might have more extractive power of active constituents than

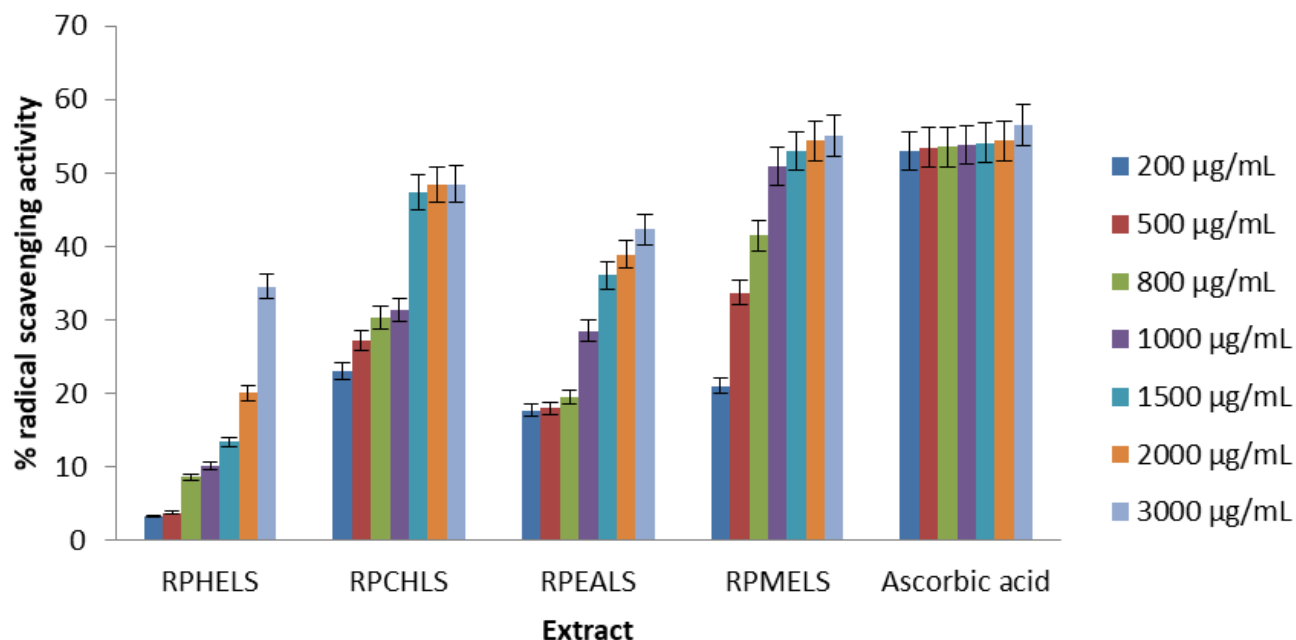


Figure 1. Percentage of radical scavenging of leaves extracts and ascorbic acid at various concentrations. RPHELS: *R. Prinoides* hexane leaves extract; RPCHLS: *R. prinoides* chloroform leaves extract; RPEALS: *R. prinoides* ethyl acetate leaves extract; RPMELS: *R. prinoides* methanolic leaves extract; Ascorbic acid in 50% methanol served as positive control.

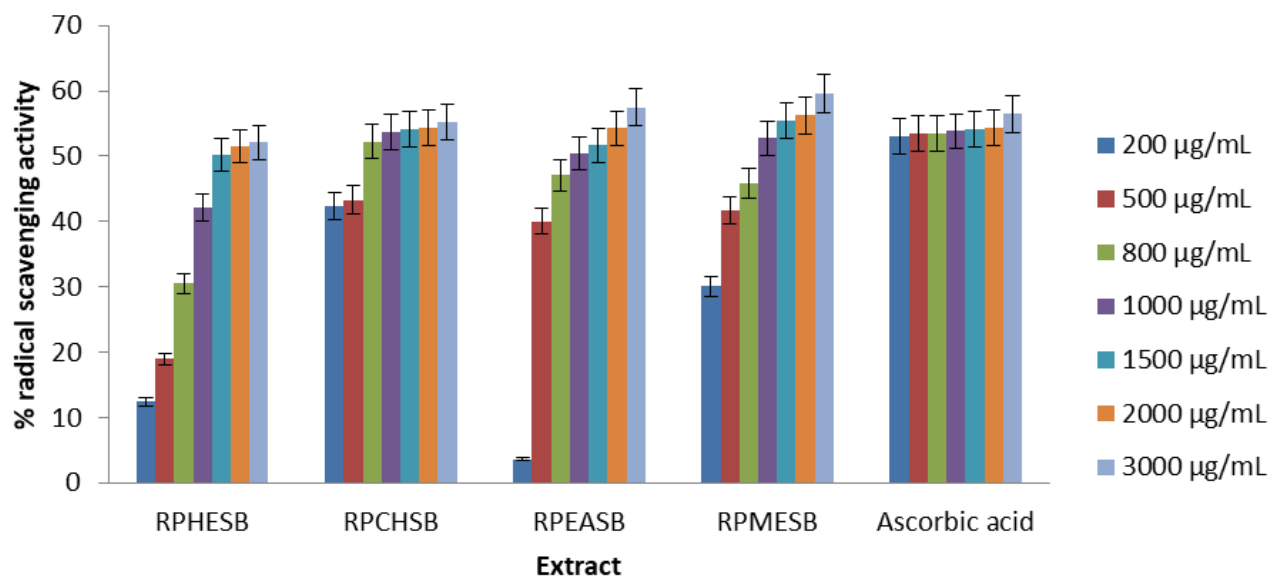


Figure 2. Percentage of radical scavenging of stem-bark extracts and ascorbic acid at various concentrations. RPHEBS: *R. prinoides* hexane stem-bark extract; RPCHSB: *R. prinoides* chloroform stem-bark extract; RPEASB: *R. prinoides* ethyl acetate stem-bark extract; RPMESB: *R. prinoides* methanolic stem-bark extract; Ascorbic acid in 50% methanol served as positive control.

the solvents used in the present study. Additionally, the collection of plant materials at different geographic locations will also play a vital role in determining the

active constituents of extracts. Methanolic and aqueous roots extracts from *R. prinoides* have also been screened for their DPPH radical scavenging activity. Their IC_{50}

Table 1. Percentage of radical scavenging activity of extracts from *R. prinoides* at various concentrations.

Extract	Concentrations ($\mu\text{g mL}^{-1}$)/ Percentage of inhibition						
	200	500	800	1000	1500	2000	3000
RPHELs	03.33 \pm 0.89	03.80 \pm 0.26	08.61 \pm 1.39	10.12 \pm 0.84	13.39 \pm 2.94	20.10 \pm 3.23	34.56 \pm 6.51
RPCHLS	23.01 \pm 3.44	27.15 \pm 5.18	30.35 \pm 1.02	31.38 \pm 0.11	47.37 \pm 4.14	48.38 \pm 4.15	48.49 \pm 3.17
RPEALS	17.71 \pm 1.02	17.96 \pm 0.82	19.48 \pm 1.50	28.51 \pm 0.62	36.05 \pm 6.26	38.94 \pm 5.75	42.33 \pm 5.88
RPMELS	21.01 \pm 3.80	33.74 \pm 1.42	41.50 \pm 2.73	50.93 \pm 5.27	52.93 \pm 2.88	54.34 \pm 2.70	55.03 \pm 3.40
RPHEsB	12.39 \pm 3.19	18.93 \pm 1.04	30.47 \pm 0.82	42.15 \pm 4.06	50.24 \pm 1.50	51.52 \pm 2.74	52.15 \pm 1.06
RPCHsB	42.37 \pm 5.65	43.30 \pm 2.98	52.23 \pm 2.46	53.64 \pm 4.42	54.06 \pm 1.41	54.41 \pm 2.17	55.22 \pm 2.48
RPEAsB	03.65 \pm 1.02	40.04 \pm 1.50	47.09 \pm 4.36	50.31 \pm 7.80	51.61 \pm 2.27	54.23 \pm 1.83	57.43 \pm 3.28
RPMEsB	30.09 \pm 5.26	41.69 \pm 2.27	45.80 \pm 2.54	52.78 \pm 6.43	55.37 \pm 3.90	56.19 \pm 3.58	59.55 \pm 2.27
Asc. acid	53.01 \pm 3.98	53.46 \pm 0.14	53.51 \pm 0.77	53.82 \pm 0.53	54.12 \pm 1.64	54.34 \pm 0.92	56.45 \pm 5.45

RPHELs: *R. prinoides* hexane leaves extract; RPCHLS: *R. prinoides* chloroform leaves extract; RPEALS: *R. prinoides* ethyl acetate leaves extract; RPMELS: *R. prinoides* methanolic leaves extract; RPHEsB: *R. prinoides* hexane stem-bark extract; RPCHsB: *R. prinoides* chloroform stem-bark extract; RPEAsB: *R. prinoides* ethyl acetate stem-bark extract; RPMEsB: *R. prinoides* methanolic stem-bark extract; Asc. Acid = Ascorbic acid in 50 % methanol served as positive control. All experiments were conducted in triplicate (n=3) and reported as the mean of three values together with standard deviation, \pm SD.

Table 2. The IC₅₀ values of various extracts of *R. prinoides* by DPPH radical scavenging assay.

S/N	Extract	IC ₅₀ ($\mu\text{g mL}^{-1}$)
1	RPHELs	>3000
2	RPCHLS	>3000
3	RPEALS	>3000
4	RPMELS	950.23
5	RPHEsB	~1500
6	RPCHsB	710.50
7	RPEAsB	~1000
8	RPMEsB	902.78
9	Asc. acid	<200

RPHELs: *R. prinoides* hexane leaves extract; RPCHLS: *R. prinoides* chloroform leaves extract; RPEALS: *R. prinoides* ethyl acetate leaves extract; RPMELS: *R. prinoides* methanolic leaves extract; RPHEsB: *R. prinoides* hexane stem-bark extract; RPCHsB: *R. prinoides* chloroform stem-bark extract; RPEAsB: *R. prinoides* ethyl acetate stem-bark extract; RPMEsB: *R. prinoides* methanolic stem-bark extract; Asc. Acid = Ascorbic acid in 50 % methanol served as positive control. All experiments were conducted in triplicate (n=3) and reported as the mean of three values together with standard deviation, \pm SD.

values were found to be 377.27 and \sim 250 $\mu\text{g mL}^{-1}$, respectively (Kimondo et al., 2019). The positive control, ascorbic acid, showed an IC₅₀ value 50.32 $\mu\text{g mL}^{-1}$ in the same assay (Kimondo et al., 2019). Additionally, the kinetics of acetylcholinesterase (AChE) inhibitory activity of aqueous extract from *R. prinoides* has previously been reported (Catherine and Edward, 2009). The IC₅₀ value for *R. prinoides* was found to be 0.201 mg mL⁻¹. The AChE inhibitory activity of *R. prinoides* was found to be higher than that of some Portuguese and Danish medicinal plants (Ferreira et al., 2006; Adersen et al., 2006). Biologically important secondary metabolites such as emodin, physcion, prinoidin, rhamnazin, geshodin and

many other emodin-derived compounds have been reported from *R. prinoides* (Van Staden and Drewes, 1994; Abegaz and Kebete, 1995). Alkaloids, flavonoids, terpenoids, anthraquinones, saponins, polyphenols, etc., classes of compounds have also been reported from various extracts of *R. prinoides* (Molla et al., 2016).

Conclusion

DPPH radical scavenging activity of hexane, chloroform, ethyl acetate and methanolic extracts from leaves and stem-bark of *R. prinoides* collected from the Kingdom of

Lesotho have been evaluated. The leaves extracts showed scavenging activity ranging from 03.33 ± 0.89 to $55.03 \pm 3.40 \mu\text{g mL}^{-1}$ while the stem-bark extracts showed relatively strong scavenging activity ranging from 03.65 ± 1.02 to $59.55 \pm 2.27 \mu\text{g mL}^{-1}$. The IC_{50} values of these extracts were also determined and were found to be in the range of 710.50 to $>3000 \mu\text{g mL}^{-1}$. *R. prinoides* finds therapeutic applications in the traditional medicine and showed promising antioxidant activity. Therefore, further studies will be useful to commercialize products from this plant.

CONFLICT OF INTERESTS

The authors declared no conflict of interests.

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Full Length Research Paper

Evaluation of cytotoxic activity of triterpenes from *Clusia studartiana*

Lavínia de C. Brito^{1,2*}, Marcia V. de Carvalho³, Vagner P. da Silva¹, Alan P. Heringer¹, Priscila M. da Silva³, Ana Paula G. A. Fontão³, Maria R. Figueiredo¹ and André Luiz F. Sampaio³

¹Laboratório de Química de Produtos Naturais, Instituto de Tecnologia em Fármacos, Fiocruz, Rio de Janeiro, RJ, Brasil.

²Central Analítica Fernanda Coutinho, Instituto de Química, UERJ, Rio de Janeiro, RJ, Brasil.

³Laboratório de Farmacologia Molecular, Instituto de Tecnologia em Fármacos, Fiocruz, Rio de Janeiro, RJ, Brasil.

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Family Clusiaceae is spread throughout tropical as well as temperate zones, presenting many triterpenes, reported as selective antitumoral agents. The aim of this study is to isolate and identify triterpenes from *Clusia studartiana* C. M. Vieira & Gomes da Silva and evaluate their cytotoxicity in melanoma (SKMEL 28) and myeloid leukemia (K562) cell lines. Thus, the hexanic extract was processed by phytochemical methods to isolate and purify the pentacyclic triterpenes: 3-oxo-friedelin (1), 3- β -hydroxy-friedelin (2) and 3-oxo-olean-12-en-28-oic-acid (3), identified by spectroscopic methods. Cytotoxicity was evaluated by MTT assay, apoptosis/necrosis by Annexin V/PI, caspase activity by FLICA in flow cytometry and P-gp modulation was measured by interference in the efflux of Rhodamine 123. From the results, triterpenes 2 and 3 showed inhibitory effect in K562 cells proliferation, and only the compound 3 was able to increase percentage of AnnexinV⁺PI⁻ in cells ($p < 0.05$), with 40% increase in caspase 3/7⁺ cells and showed inhibition on P-gp activity.

Key words: Apoptosis, caspase, Clusiaceae, *Clusia studartiana*, cytotoxicity, multidrug resistance.

INTRODUCTION

The family Clusiaceae is spread in temperate and tropical areas regions with 14 genera distributed in 800 species, including trees and shrubs, hemiepiphytes, epiphytes and lianes (Stevens, 2017). The plants of this family are cultivated for various purposes, such as production of noble wood, edible fruits and to bioactive compounds for the pharmaceutical industries (Souza et al., 2013; Kotraswamy et al., 2016). In ethnomedicine, this family has been used for the treatment of numerous diseases,

including inflammation, infection and cancer (Melo et al., 2014). In particular, many pentacyclic triterpenes have already been found in this family (Jamila et al., 2015; Duprat et al., 2017; Ribeiro et al., 2019).

The number of studies using natural products as chemotherapeutic agents has increased exponentially in recent years and pentacyclic triterpenes are often described with a wide range of pharmacological activities. These compounds are divided into three structural types:

*Corresponding author. E-mail: laviniabrito@yahoo.com.br.

oleanane, ursane and friedelane (Salvador et al., 2017). In this context, oleanane, ursane and friedelane-types exhibit inhibitory activity against various intra and extracellular targets in eukaryotic cells and proteins that have participation in different processes such as cellular development, differentiation, inflammation, angiogenesis, apoptosis and metastasis (Iqbal et al., 2018; Peron et al., 2018; Ren and Kinghorn, 2019). Thus, they are considered as multitarget agents, acting through several signaling pathway, involved in cancer progression and survival. This ability makes them interesting for the development of new antineoplastics agents (Salvador et al., 2017; Ren and Kinghorn, 2019). In addition, pentacyclic triterpenes present selective cytotoxicity, capable to distinguish between tumor and non-tumor-cells, possibly associated with their ability to modulate tumor microenvironment and the immune system (Kamble et al., 2014). Therefore, these compounds have low toxicity in normal cells and high efficiency on cancer cells, reported in many *in vitro* and *in vivo* studies, including clinical trials (Gill et al., 2016; Zhang et al., 2014).

In cancer chemotherapy, the occurrence of multidrug resistance (MDR) is one of the main obstacles to the successful treatment. MDR has various molecular mechanisms such as the increase of drug efflux, activation of detoxification systems, alteration of drug targets, changes in cell cycle, activation of DNA repair and escape of apoptosis (Ye et al., 2019). In this context, P-glycoprotein (P-gp), a drug transporter more frequently related to MDR pumps substrates out of cells by an ATP-dependent mechanism, promoting decrease in the intracellular accumulation of many antitumor drugs to sub-therapeutic levels, reducing or abolishing the efficacy of the treatment. Some MDR reversal agents have been developed and participated in clinical trials. However, most of them failed due to severely adverse effects (Liu et al., 2015; Robey et al., 2018). In this context, natural products such as terpenoids, alkaloids and flavonoids are a source of promising anti-cancer compounds and also as potential modulators of MDR (Kumar and Jaitak, 2019), through its combination with chemotherapeutics agents (Mahdizadeh et al., 2016). Considering the chemical profile and antineoplastic activity already described for the Clusiaceae family, the present study aimed to evaluate the chemical composition, cytotoxicity, pro-apoptotic nature and the effects of MDR with the isolated compounds of *Clusia studartiana* C.M.Vieira and Gomes da Silva

MATERIALS AND METHODS

Plant material

Aerial parts of *C. studartiana* C.M. Vieira & Gomes da Silva (Clusiaceae) were collected at the Ecological Reserve of Macaé de Cima, Sophronites Site, Atlantic Forest, Nova Friburgo, Rio de Janeiro RJ (22°23'56.8"S 42°30'34.3"W), identified by Dr Marcos

Nadruz. The specimen was deposited in the Herbarium of the Botanic Garden of Rio de Janeiro, number RB 336999. The species is registered in the Genetic Patrimony (SISGEN) under the number AB5D582.

Extraction, isolation and purification of pentacyclic triterpenes

The extract of the aerial parts (1.40 kg) of *C. studartiana* was prepared by dynamic maceration, at room temperature, with hexane until exhaustion. The extract was filtrated and evaporated under reduced pressure to obtain the hexanic extract (26.31 g). During concentration of the hexanic extract, an amorphous solid (10.14 g) was obtained. This solid was chromatographed on silica gel, using mixtures of solvents with increasing polarities (cyclopentane, cyclopentane/ethyl acetate, ethyl acetate and methanol). The fractions were combined according to their chromatographic profile by thin layer chromatography (TLC) and visualized with Godin detection reagent. After successive recrystallizations, it was obtained the pentacyclic triterpenes: 3-oxo-friedelin (3.29 g), 3 β -hydroxy-friedelin (0.23 g), 3-oxo-olean-12-en-28-oic acid (5.78 g) pures.

The purity and identification of these compounds were determined by GC-MS and/or the structural characterization by one- and two-dimensional methods in ¹H- and ¹³C-NMR (500MHz). The molecular formula of the acid isolated was determined through direct insertion in a Bruker microTOF-QII mass spectrometer by accurate mass and algorithmic analysis of its isotopic pattern (Bruker SmartFormula 3D True Isotopic Pattern). This acid was methylated with diazomethane (Black, 1983). For pharmacological assays, a pentacyclic triterpenes stock solution (40mM) was performed using cell culture grade DMSO (Sigma), diluted to working concentrations, in cell culture medium minutes prior treatment.

Cellular cytotoxicity assay with colorimetric method of the MTT

For this assays, human myeloid leukemia cells (K562) and human melanoma cells (SK-MEL-28) were respectively cultured in RPMI 1640 or DMEM supplemented with 10% FBS, 100 U/I penicillin, 0.1 mg/mL streptomycin and 0.05 mg/mL gentamicin and maintained in log growth phase. Twenty-four hours before treatment with samples, 100 μ L of the cell suspension (5×10^4 cells/ml) was added to 96-well plates and maintained in a 5% CO₂ atmosphere at 37°C. Treatment with the pentacyclic triterpenes was performed in a single (10 μ M) or multiple concentrations (0.01 to 100 μ M), each in triplicate. Negative control consisted of cells incubated with vehicle (0.25% of cell culture grade DMSO in medium; SIGMA), and staurosporine (SIGMA; 1 μ M) as inhibition control. After 48 h, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; SIGMA) was added and the plate maintained in CO₂ incubator for 4 h. After the incubation time, the supernatant was aspirated and formazan crystals, formed by cellular activity, solubilized with isopropyl alcohol (SIGMA) (Mosmann, 1983; Spinner, 2001). Absorbance was measured at 540 nm wavelength in a microplate reader (Victor X5; Perkin Elmer USA)

Determination of apoptosis/necrosis by flow cytometry analysis (Annexin V/propidium iodide)

For apoptosis assays, 105 cells K562 cells were seeded (1 ml at 105 cells/ml) in a 24-well plate and incubated in a 5% CO₂ atmosphere at 37 °C. Twenty-four hours after seeding, cells were treated in duplicate with test samples (10 and 100 μ M) for 4 h. Negative control consisted of cells incubated with vehicle (0.25% of cell culture grade DMSO in medium; SIGMA), and staurosporine

(Sigma; 5 μM) was used as positive control. Next, cells were collected in 4 mL tubes, centrifuged and washed with sterile phosphate buffered saline (PBS). After centrifugation, the supernatant was discarded and cells resuspended in 100 μl of binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl_2 , pH 7.4.) containing Annexin V, as recommended by the manufacturer (Annexin V-Alexa Fluor 488 – Molecular Probes). Cells were incubated at room temperature (20 min) and protected from light. Then 400 μl of binding was added and contents were transferred to flow cytometry tubes kept on ice. It was added 2 μl of a propidium iodide (PI; SIGMA) solution (250 $\mu\text{g/ml}$ in binding buffer) at the time of acquisition, (within 5 min) to avoid false positive due to overexposure with PI. Data acquisition was performed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest™ software (Becton Dickinson). Forward- and side-scatters were set to exclude debris. At least 104 cells were analyzed per sample. Apoptosis status was classified by cell staining profile: early apoptosis (Annexin V+/PI-); late apoptosis (Annexin V+/PI+); necrosis (Annexin V-/PI+).

Determination of caspase activity by flow cytometry analysis (FLICA)

For this procedure, 10^5 cells K562 cells were seeded (1 ml at 10^5 cells/ml) in a 24-well plate and incubated in a 5% CO_2 atmosphere at 37°C. Twenty-four hours after seeding, cells were treated in duplicate with samples (100 μM), for 6 h and centrifuged. Negative control consisted of cells incubated with vehicle (medium containing 0.25% of cell culture grade DMSO; SIGMA) or staurosporine (Sigma; 5 μM), as positive control. Supernatant was discarded and the cells resuspended in 100 μl culture medium and FLICA solution was added according to the manufacturer's instructions (Vybrant FAM Caspase-3 and -7 Assay Kit - Molecular Probes); cells were incubated for 60 min at 37°C in a CO_2 protected from light. After the incubation time, cells were washed twice and resuspended in wash buffer and fixed by adding the fixing buffer. Experiments were performed twice in duplicate with a total of 10^4 events acquired. Data acquisition was performed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and analysis using CellQuest™ software (Becton Dickinson). Forward- and side-scatters were set to exclude debris.

Monitoring assay of P-gp activity

The methodology for monitoring P-gp activity was adapted from the procedure described by Maia et al. (1996). K562-Lucena 1 (a K562 resistant cell line with multidrug resistant (MDR) characteristics) was cultured in RPMI 1640 medium at 37°C in a 5% CO_2 atmosphere. In a U-bottom 96-well plate, 2×10^5 cells/well were incubated with vehicle as negative control (medium containing 0.25% of cell culture grade DMSO; SIGMA), samples (100 μM) or verapamil (10 μM ; positive control for P-gp inhibition) in the presence of rhodamine 123 (200 ng/ml) for 45 min at 37°C. After incubation, cells were centrifuged, the supernatant discarded and cells washed by adding ice-cold PBS (200 μl). Cells were maintained on ice until data acquisition in a FacsCalibur flow cytometer. Experiments were performed twice in triplicate, a total of 10^4 events were acquired and Rhodamine 123 (Rh123) fluorescence monitored on FL-1 channel and analysis performed by CellQuest program.

Statistical analysis

The results were expressed as the mean, plus or minus the

standard error of the mean (SEM). Statistical analysis was performed by One Way ANOVA variance analysis, followed by Newman Keuls test and considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Three compounds were isolated and purified by phytochemical methods from *C. studartiana* namely 3-oxo-friedelin (1), 3- β -hydroxy-friedelin (2), 3-oxo-olean-12-en-28-oic-acid (3). The compound methyl olean-12-en-3-oxo-28-oate (4) was obtained after derivatization of acid (3) by diazomethane (Black 1983) (Figure 1). Triterpenes were characterized by one- and two-dimensional methods in ^1H - and ^{13}C -NMR and confirmed by comparison with spectral data from the literature (Queiroga et al., 2000; Mkounga et al., 2016).

These pentacyclic triterpenes were tested on K562 and SK-MEL-28 cell lines for their ability to inhibit *in vitro* cell proliferation. We have adopted the strategy of testing molecules at a single concentration (10 μM) and, if an inhibition above 30% was observed, the molecule was further tested in multiple concentrations. This methodology follows the NCI strategy for drug discovery (Shoemaker, 2006). Among compounds tested at 10 μM , the compounds 2 and 3 showed inhibitory effect (41.59 % and 34.27 %, respectively) on K562 cells, while the compounds 1 and 4 showed inhibitory effect lower than 30%. All of these compounds have had no cytotoxicity on SK-MEL-28 cells (Table 1).

Compounds (10 μM) were tested for cell proliferation, as described in methodology with Staurosporine (STAU; 1 μM) as a positive control. For calculation purposes, cells cultured in medium containing 0.25% DMSO were used as 100% proliferation. Percentages of inhibition are shown as mean \pm standard error of mean (SEM) of a triplicate.

Compounds 2 and 3 caused growth inhibition on K562 myeloid leukemia cells, in a dose response manner with IC_{50} of 8.92 μM and 26.67 μM respectively, with a maximum inhibition of 89.32% triggered by compound 2 at 100 μM (Figure 2). Due to its performance and good IC_{50} on K562 cell line, we have also tested compound 2 on K562 Lucena cell line proliferation (MDR phenotype). We have observed, as expected, an increase in the compound 2 IC_{50} from 8.92 μM on K562 cell line to 23.69 μM on K562 Lucena cell line. Cells cultured in medium containing 0.25% DMSO were used as controls for 100% proliferation, points represent mean \pm standard error of mean (SEM) of six replicates from two independent experiments. Curves were analyzed using Prism 5 software for IC_{50} determination. Between these compounds only 3 was able to modulate P-gp activity on K562 Lucena, a K562 resistant cell line with multidrug resistant (MDR) characteristics. This acid increased retention of rhodamine 123, observed through fluorescence of labelled cells. This suggests that this terpenoid could alter MDR phenotype of tumor cells

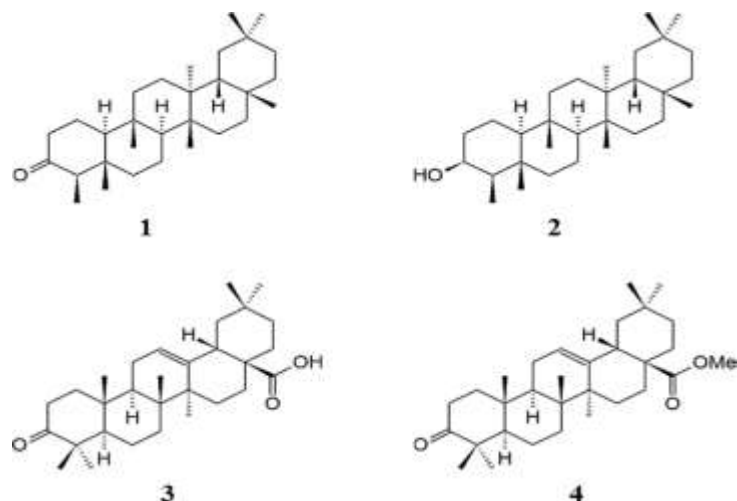


Figure 1. Chemical structure of the pentacyclic triterpenes 3-oxo-friedelin (1), 3-β-hydroxy-friedelin (2), 3-oxo-olean-12-en-28-oic-acid (3) previously isolated from the aerial parts of *C. studartiana* and methyl olean-12-en-3-oxo-28-oate (4).

Table 1. Effects of compounds isolated from *C. studartiana* on the inhibition of tumor cell proliferation.

Compound	% INHIBITION	
	SKMEL28	K562
STAU	76.45 ± 2.37	68.87 ± 0.38
1	5.12 ± 1.98	12.06 ± 8.62
2	6.32 ± 3.75	41.59 ± 8.41
3	6.33 ± 1.09	34.27 ± 4.06
4	4.64 ± 0.82	12.54 ± 3.08

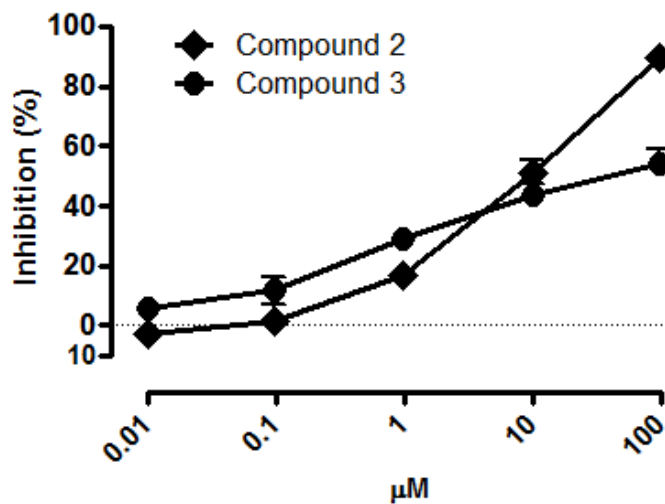


Figure 2. Effect of compounds 2 and 3 on K562 cell proliferation. Cells were cultivated and treated with compound 2 (♦) and 3(●) in increasing concentrations (0.01 μM to 100 μM) for proliferation assessment using MTT technique, as described in methods.

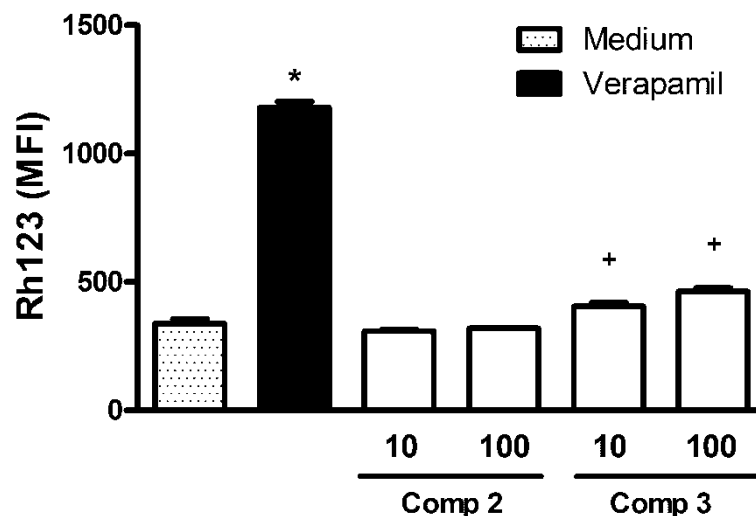


Figure 3. Evaluation of P-gp activity in K562-Lucena 1 cells treated with compounds 2 and 3. Cells were incubated (45 min) with rhodamine 123 (200 ng/ml) concomitant with samples (10 μ M and 100 μ M), verapamil (10 μ M; inhibition control) or medium containing 0.25% DMSO, as negative control.

(Figure 3). Further studies are necessary to better understand this aspect of this terpenoid.

Samples were processed and the mean fluorescence intensity (MFI) was evaluated by flow cytometry. Columns represent mean + SEM of a triplicate from a representative experiment. Differences were considered significant when $p < 0.05$ after statistical analysis by One Way ANOVA followed by Newman Keuls. * means $p < 0.05$, when compared to cells treated with medium + means $p < 0.05$, when compared to cells treated with medium and verapamil.

Other structure related to pentacyclic triterpenes, such as betulinic, oleanolic and pomolic acids, present inhibitory effect on K562 proliferation (Fernandes et al., 2003), suggesting that triterpenes evaluated in this study may have a mechanism of action similar of those terpenoids (Kumar et al., 2018). Li et al. (2010) have described that friedelin and other structure-related molecules inhibit HL-60 proliferation. Moreover, Huang et al. (2006) demonstrated that compound 3 presented cytotoxicity against several tumoral cells *in vitro* (HELF, Ketr-3, Bel7402, A549, HT-1080, PC-3M, KB and KB-V cells) and *in vivo* (B16-BL6), reinforcing the antitumoral component of compound 3 observed on K562 cells. Herein we have shown, for the first time, the cytotoxic activity of compound 3 on K562 leukemia cells and its ability to modulate the P-gp activity on K562 Lucena.

Samples were processed in duplicate and analyzed for Annexin V expression and PI labelling by flow cytometry. Apoptosis status classified by cell staining profile: early apoptosis (Annexin V⁺/PI⁻); late apoptosis (Annexin V⁺/PI⁺); necrosis (Annexin V⁻/PI⁺). Percentages of a representative sample from a valid experiment are

shown. We have analyzed apoptosis and caspase activation to better understand the mechanisms of growth inhibition on K562 cells by compounds 2 and 3. As shown in Figure 4, compound 3, but not compound 2, was able to change annexin V expression in K562 cells in a single cell level. We have observed that compound 3 treatment was able to increase the percentage of annexin V⁺ cells (Figure 5B); however these cells were not stained with PI, suggesting that cells were in early apoptosis (annexin V⁺/PI⁻; Figure 5A). However, compound 2 failed to change apoptosis or necrosis after treatment. This suggests a different mechanism of action for this compound, probably a delayed caspase activation or a caspase-independent mechanism (Giampazolias et al., 2017).

Samples were processed in duplicate and analyzed by flow cytometry and apoptosis status classified by cell staining profile: early apoptosis (Annexin V⁺/PI⁻); late apoptosis (Annexin V⁺/PI⁺); necrosis (Annexin V⁻/PI⁺). Columns represent mean \pm SEM, from two independent experiments. Differences were considered significant when $p < 0.05$ after statistical analysis by One Way ANOVA variance analysis, followed by Newman Keuls. * means $p < 0.05$, when compared to cells treated with medium.

Pentacyclic triterpene 2 failed to trigger apoptosis or necrosis in K562 cells. This data were supported by evaluation of caspase 3/7 activity as there was no increase in activity after 2 treatment, suggesting that this triterpenoid may trigger a delayed caspase activation or, more interestingly, causing cell death in a caspase-independent manner (Figure 6). On the other hand, we have observed a raise in 40% of caspase 3/7⁺ in 3

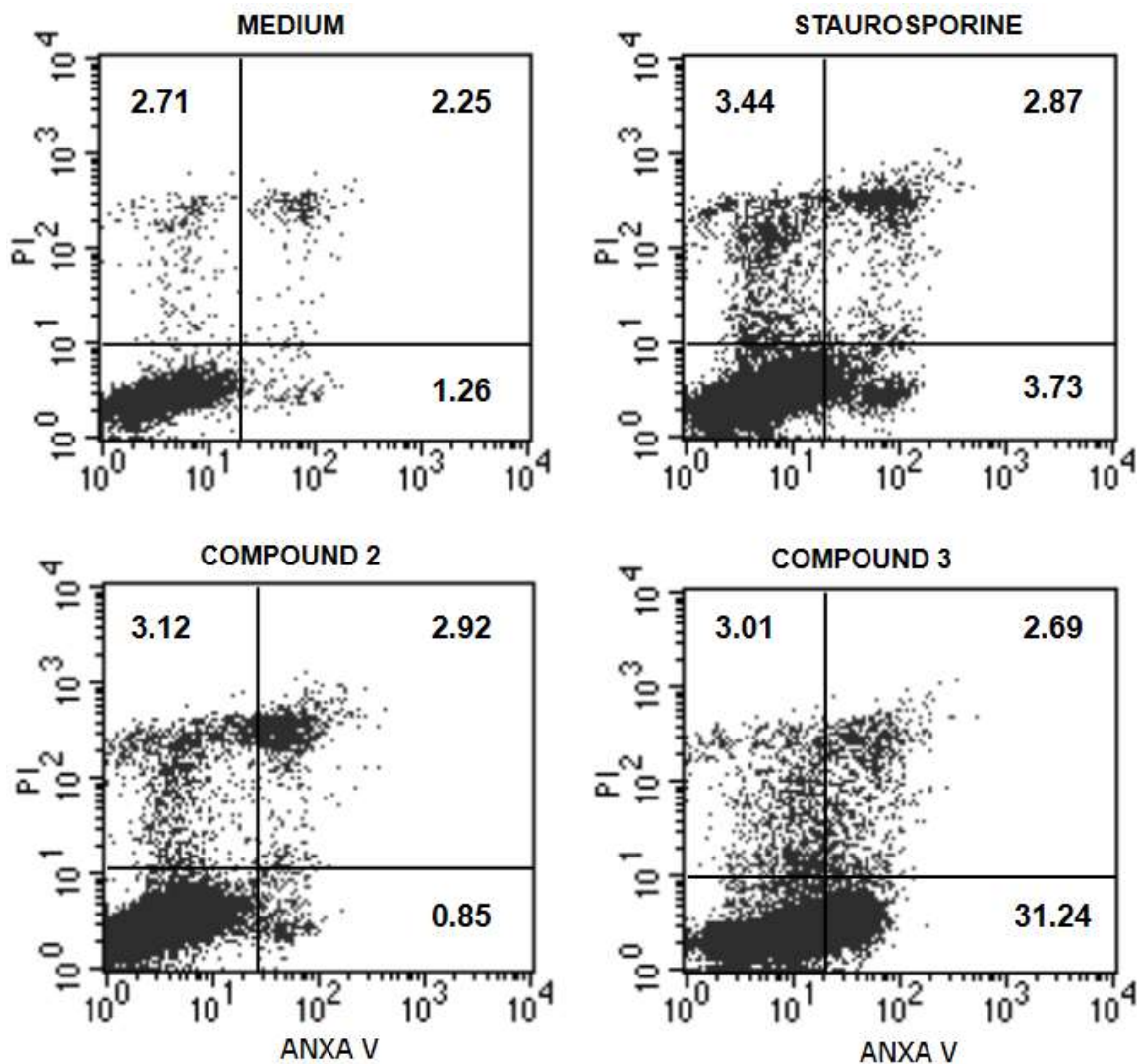


Figure 4. Evaluation of annexin V/PI expression on K562 cells, treated with compound 2 and 3 in single concentration (100 μ M). For these experiments, staurosporin (STAU; 1 μ M) and cells cultured in medium containing 0.25% DMSO (MediumM) were used as controls.

treated cells (from 2.03% in medium to 2.85% after treatment), when compared to cells cultured in medium alone, in agreement with proliferation and annexin V expression.

Triterpenoids such as betulinic acid, friedelin, their derivatives and structure related molecules have antitumor activities (Peron et al., 2018; Kumar et al., 2018). These molecules may trigger intrinsic apoptosis via different pathways. Moreover, betulinic acid may also trigger apoptosis in a caspase-independent fashion (Kumar et al., 2018). Herein, we have described that compound 2 have a inhibitory activity on cell proliferation (Figure 2) without changes on annexin V expression in the outer membrane (Figure 5) and caspase 3/7 activity (Figure 6). This suggests that compound 3 may activate a different pathway for apoptosis induction, independent of

caspase activation, that still need to be investigated. On the other hand, similarly to observed after treatment of cancer cells with betulinic acid or friedelin-like molecules (Raghuvar et al., 2005; Subash-Babu et al., 2017; Kumar et al., 2018), compound 3 increased annexin V expression and induced a discrete increase in caspase 3/7 activity, suggesting that this compound may trigger the mitochondrial pathway. The exact mechanisms underpinning apoptosis triggered by compound 3 are still under investigation. Samples were processed and analyzed by flow cytometry, and results shown as mean \pm SEM of a duplicate. Differences were considered significant when $p < 0.05$ after statistical analysis by One Way ANOVA variance analysis, followed by Newman Keuls. ** means $p < 0.05$, when compared to cells treated with medium.

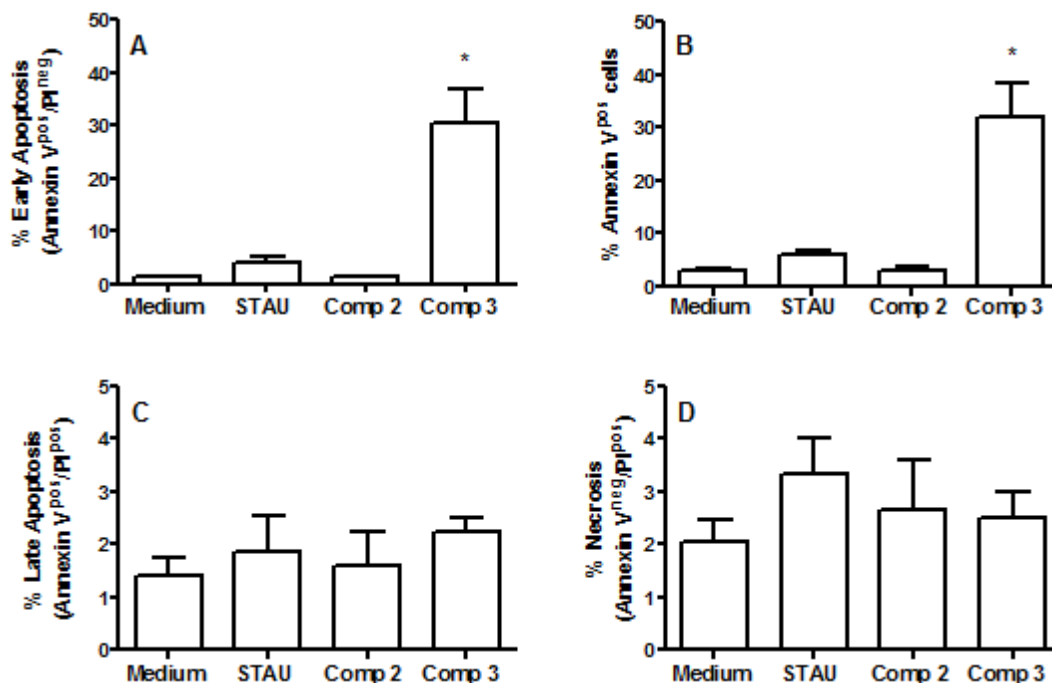


Figure 5. Evaluation of annexin V/PI expression on K562 cells, treated with compound 2 and 3 in single concentration (100 μ M). For these experiments, staurosporin (STAU; 1 μ M) and cells cultured in medium containing 0.25% DMSO (MEDIUM) were used as controls.

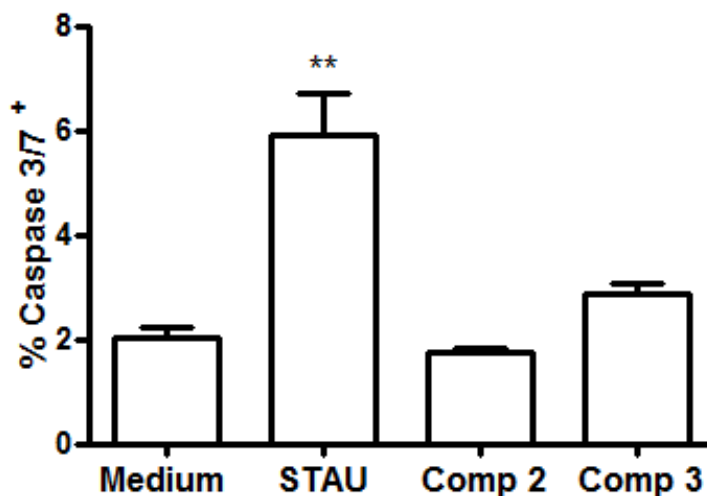


Figure 6. Evaluation of Caspase 3/7 activity on K562 cells, treated with compounds 2 and 3 in single concentration (10 μ M), using staurosporin (STAU; 1 μ M) as a positive control and cells cultured in medium plus 0.25% DMSO (Medium) as negative control.

Conclusion

In conclusion, compound 3 caused cell growth inhibition in human myeloid leukemia cells (K562); it was able to induce apoptosis, by a mechanism that involved the

activation of caspases 3/7 and promoted discrete inhibition of P-gp activity, an important aspect of MDR phenotype. Further studies are needed for a better understanding of apoptosis-induction processes triggered by this compound.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Modulatory effects of *Momordica balsamina* on Th1/Th2 cytokine profiles in immune-challenged rats

Iman H. Abdoon^{1*}, Bashier Osman¹, Maowia M. Mukhtar² and Hatim Ali Elsheikh³

¹Department of Pharmacology, Faculty of Pharmacy, University of Khartoum, Khartoum, Sudan.

²Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan.

³Department of Clinical Pharmacology, College of Medicine, Taif University, Taif, Kingdom of Saudi Arabia.

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There is an ever-growing interest to identify plants that boost the immune system functions. The objective of the present study was to evaluate the immunomodulatory effects of *Momordica balsamina* (MB) leaves extract in BCG-immunized rats. Thirty rats were randomly divided into five groups (n = 6). MB extract was suspended in 1% carboxymethyl cellulose (CMC). Firstly, animals were challenged subcutaneously with 0.05 ml BCG. The first group (vehicle control group) received 1% CMC (100 mg/kg body weight; p.o.), and the second group (positive control group) was provided with levamisole (18 mg/kg body weight; p.o.). The remaining groups (test groups) were dosed orally with different doses of MB extracts (50, 100 and 200 mg/kg body weight) for 14 consecutive days. Blood samples were collected on day 0, 7 and 14, and then plasma samples were analyzed for Th1 cytokines (TNF- α and IFN- γ) and Th2 cytokines (IL-10 and TGF- β), using cytokine specific enzyme-linked immunosorbant assay (ELISA). MB extracts significantly increased ($p \leq 0.01$) IFN- γ production in a dose- and time-dependent manner and elicited significant increase ($P \leq 0.05$) of TNF- α level at the dose 200 mg/kg after 14 days. Low doses (50 and 100 mg/kg) significantly increased ($p \leq 0.05$) TGF- β levels while a high dose (200 mg/kg) significantly ($p \leq 0.05$) reduced TGF- β levels in a time-dependent manner. No significant changes were observed on IL-10 level after plant extract treatment. MB shows immunostimulatory effects and significantly activates cell-mediated immunity.

Key words: *Momordica balsamina*, cell-mediated immunity, Th1 cytokine, Th2 cytokine.

INTRODUCTION

Interest in medicinal plants has recently burgeoned due to increased efficiency of new plant-derived drugs coupled with rising cost of conventional medicines (Karali et al., 2011; Siveen and Kuttan, 2012). In nature, various medicinal plants are believed to promote positive health

and enhance the natural resistance of the body to various human ailments. A large number of medicinal plants have been claimed to possess immunomodulatory activities (Kumar et al., 2012; Eze et al., 2017; Ahmad et al., 2018; Shruthi et al., 2018). Medicinal plants as botanical

*Corresponding author. E-mail: imanabdoon@yahoo.com. Tel: 00249125053862.

immunomodulators can provide alternative potential to conventional chemotherapy for a variety of immune disorders such as autoimmune diseases or conditions of impaired immune response (Mainardi et al., 2009). The therapeutic potential of plant-based immunomodulators and their curative properties have been highlighted by many researchers. In recent years, there has been growing interest to use herbal medicines as multi-component agents to modulate the immune system for prevention of infections and neuroinflammation (Jantan et al., 2015). Plant-derived natural products such as polysaccharides, flavonoids, alkaloids, sesquiterpene lactones and triterpenes have received considerable attention in recent years due to their diverse pharmacological properties. Phytochemicals such as flavonoids, polysaccharides, alkaloids, lectins, glycosides, phenolic compounds, tannins, saponins, terpenoids, sterols have been reported to modulate the immune system (Jantan et al., 2015; Venkatalakshmi et al., 2016; Oh et al., 2018).

The regulation of immune responses depends on the local production of a number of cytokines which are a diverse group of proteins that act as mediators between cells. T-cells participate in a wide range of immune responses through cytokine network. IL-2 differentiates CD4+ T-cells into two subsets of cells, T helper 1(Th1) and T helper 2 (Th2) cells. Th1 cells stimulate cell-mediated immunity through secretion of Th1 cytokines (TNF- α and IFN- γ) which activate macrophages to eradicate intracellular microbes. Th2 subset of cells stimulates antibody-mediated immunity through secretion of Th2 cytokines (IL-4, IL-5 and IL-10) (Romagnani, 2000).

Momordica balsamina, also known as Balsam Apple, belongs to the family Cucurbitaceae. It is annual or short-lived perennial herbaceous climber with yellow-orange warty fruit when ripe. The plant is widely found/cultivated throughout the drier part of tropical and subtropical Africa, Asia and Australia (Welman, 2004; Behera et al., 2011). Different parts of *M. balsamina* (leaves, fruits, seeds) contain various phytochemicals such as alkaloids, flavonoids, glycosides, steroids, terpenes, cardiac glycoside and saponins (Thakur et al., 2009; Nagarani et al., 2014). *M. balsamina* has been used as a traditional folk medicine in many countries, and it is used medicinally as anti-plasmodial, shigelloidal, anti-diarrheal, anti-septic, anti-bacterial, anti-viral, anthelmintic, anti-inflammatory, hypoglycemic, antioxidant, analgesic and hepatoprotective agent (Thakur et al., 2009; Ramallete et al., 2010; Nagarani et al., 2014).

In recent years there has been an upsurge in the clinical uses of plant-derived natural substances to strengthen natural immunity against various ailments. The aim of this study is to evaluate the immunomodulatory effect of methanolic extract of *M. balsamina* leaves in rodent model of BCG-immunized rats.

MATERIALS AND METHODS

Animals and maintenance

All animal work was carried out at the Faculty of Pharmacy, University of Khartoum (Khartoum, Sudan) in accordance with Institutional Animal Welfare Guidelines; and all experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Faculty of Pharmacy - University of Khartoum, Sudan). Albino rats (average weight 180 grams) of either sex were obtained from the animal house of the Faculty of Pharmacy, University of Khartoum, Sudan. They were housed in standard plastic cages, under standard environmental conditions of temperature (18-26°C), light (12 h light/dark cycle) and controlled humidity. They had free access to standard diet and water *ad libitum* (Elahi et al., 2017).

Preparation of plant extract

M. balsamina was obtained from Northern Kordofan in Western Sudan. It was identified and authenticated by the Department of Medicinal and Aromatic Plants at the National Centre for Research, Khartoum, Sudan. Briefly, leaves of *M. balsamina* were air-dried at room temperature to avoid possible decomposition of the plant constituents and then pulverized into coarse powder. The plant's powder was macerated in 80% methanol at room temperature for 24 h with occasional shaking. The extract filtrate was concentrated under reduced pressure, using the rotary vacuum evaporator, and then dried to a solid mass under air at room temperature (Jones and Kinghorn, 2006).

Experimental protocol

Thirty rats (average body weight 180 g) were randomly divided into five groups (n = 6). *M. balsamina* extract was suspended in 1% carboxymethyl cellulose. Firstly, all animals were immunized subcutaneously with 0.05 ml BCG on day 0 to challenge the immune system. The first group (vehicle control group) received 1% carboxymethyl cellulose (100 mg/kg body weight; p.o.), and the second group (positive control group) was provided with levamisole (18 mg/kg body weight; p.o.) for 14 consecutive days (Stogaus and King, 1995). The remaining three groups (test groups) were dosed orally, for 14 consecutive days, with three doses (50, 100 and 200 mg/kg body weight) of *M. balsamina* extracts, suspended in 1% carboxymethyl cellulose.

Collection and preparation of plasma samples for assay

For quantitative measurement of cytokines in plasma, about three milliliter of blood samples were collected from the retro-orbital plexuses of individual rat under mild ether anesthesia on day 0 (just before dosing and immunization), day 7 and day 14. Plasma samples were separated after centrifugation and then stored at -20°C until further analysis (Elhag et al., 2011).

Measurement of Th1 cytokines in plasma

Levels of TNF- α and IFN- γ (Th1 cytokines) in plasma were quantified, using ELISA kits (Peprotech Company, USA), according to the manufacturer's instructions. Briefly, 96-well microtiter plates (Nunc/Thermo scientific industry, Denmark) were coated overnight at room temperature with capture antibody, washed and then

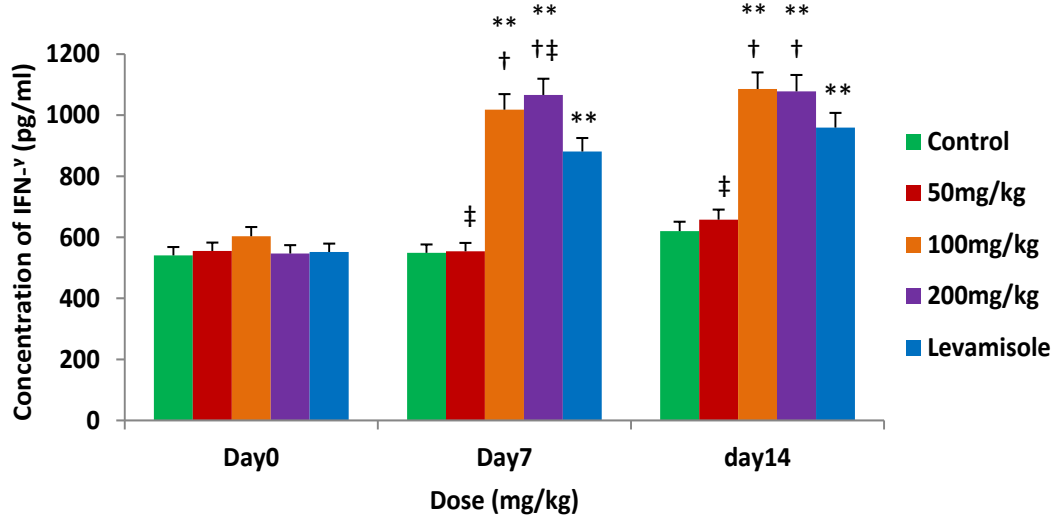


Figure 1. Dose-dependent effects of *M. balsamina* on IFN- γ levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. **, $p \leq 0.01$ versus control. †, $p \leq 0.05$ versus 50 mg/kg. ‡, $p \leq 0.05$ versus levamisole.

blocked by addition of block buffer (1% fetal calf serum in phosphate buffer solution) for one hour at room temperature. Samples and series of diluted standards were immediately added in duplicate to the plates and incubated at room temperature for at least two hours. After washing, biotin-conjugated detection antibody was added and then incubated at room temperature for two hours. After 30 min incubation of avidin-HRP conjugate at room temperature, ABTS (2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); purchased from Sigma-aldrich, USA) liquid substrate was added and then incubated at room temperature for color development. The reactions were stopped after 15 min and the color was immediately measured at 405 nm, using automated ELISA plate reader. TNF- α and IFN- γ levels were calculated from calibration curves of diluted TNF- α and IFN- γ standard concentrations, respectively. Concentrations of the cytokines were determined in pg/ml.

Measurement of Th2 cytokines in plasma

For quantitative measurement of TGF- β 1 and IL-10 levels (Th2 cytokines), Rat TGF- β 1 and IL-10 ELISA kit (Bender Medsystem Company/eBioscience) were used, following the manufacturer's protocol. In brief, measurement of TGF- β required acidification of plasma samples for activation of inactive latent TGF- β before starting the test procedure. Diluted samples and standards were added in duplicate to pre-coated 96-well microtiter plates (coated with anti-rat TGF- β 1mAbs or anti-rat IL-10 mAbs) and then incubated for two hours at room temperature. After washing, biotinylated detection antibodies were added and incubated for one hour, followed by the addition of streptavidin-HRP and further incubation for one hour. Thereafter, TMB (tetramethyl-benzidine; purchased from eBioscience) substrate was added and incubated for 30 min at room temperature for color development. The reactions were stopped with 1 M phosphoric acid, and the color intensity was measured at 450 nm, using a microtiter plate reader. TGF- β and IL-10 concentrations were calculated from standard curves of TGF- β and IL-10 standard solutions, respectively. Concentrations of the cytokines were determined in pg/ml.

Statistical analysis

Data were expressed as Mean \pm S.D. Levels of difference between all groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. *P*-values < 0.05 were considered as statistically significant.

RESULTS

Effects of *M. balsamina* leaf extract on IFN- γ level in BCG-immunized rats

Evaluation of immunomodulatory effect of *M. balsamina* extract on Th1 cytokine revealed that the plant extract significantly increased ($p \leq 0.01$) IFN- γ levels in a dose/time-dependent manner. Daily administration of 100 and 200 mg/kg of *M. balsamina* extract exhibited a significant increase ($p \leq 0.01$) of IFN- γ level after 7 and 14 days when compared to control. Interestingly, IFN- γ levels at doses of 100 and 200 mg/kg after 7 or 14 days were greater than that observed after treatment with levamisole (positive control). *M. balsamina* leaf extract increased IFN- γ level in BCG-immunized rats as shown in Figures 1 and 2.

Effects of *M. balsamina* leaf extract on TNF- α level in BCG-immunized rats

In order to delineate the immunomodulatory effects of *M. balsamina* extract, the levels of TNF- α (Th1 cytokine) were measured after administration of three doses of the extract. Daily administration of *M. balsamina* extract

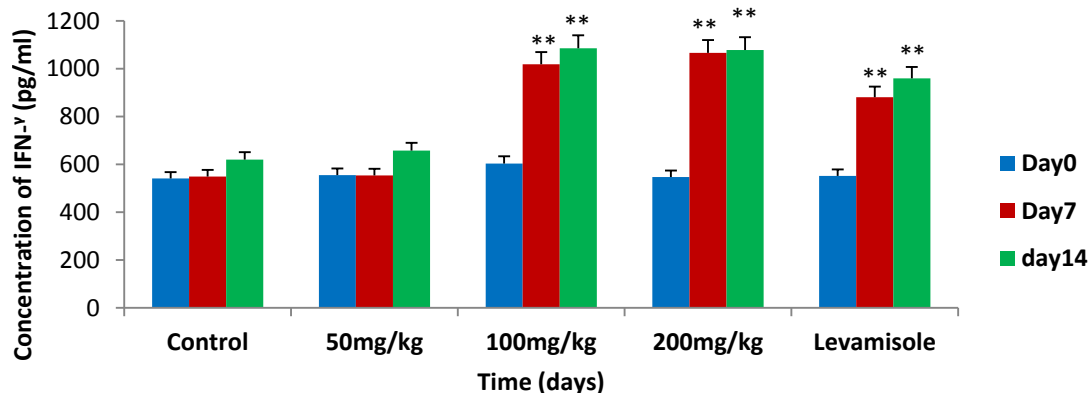


Figure 2. Time-dependent effects of *M. balsamina* on IFN- γ levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. **, $p \leq 0.01$ versus day 0

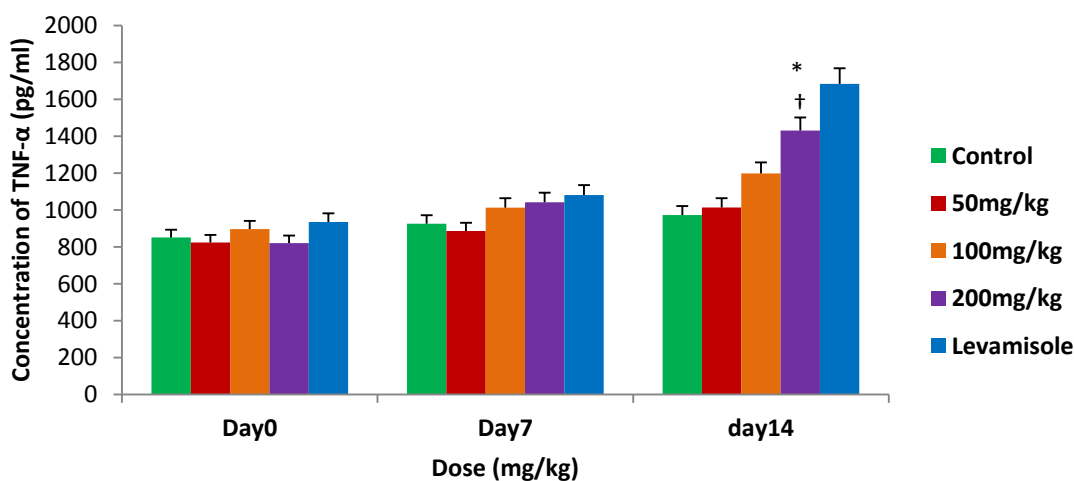


Figure 3. Dose-dependent effects of *M. balsamina* on TNF- α levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. *, $p \leq 0.05$, versus control. †, $p \leq 0.05$ versus 50 mg/kg.

appears to exert dose-dependent increase of TNF- α levels after 14 days. Interestingly, 200 mg/kg of *M. balsamina* extract showed a significant increase ($P \leq 0.05$) of TNF- α level after 14 days when compared to control (Figure 3); and exhibited a significant increase ($P \leq 0.05$) of TNF- α level in a time-dependent manner. In administration of *M. balsamina* extract, neither 50 mg/kg nor 100 mg/kg dose affected TNF- α level with respect to time (Figure 4). *M. balsamina* leaf extract increased TNF- α level in BCG-immunized rats.

Effects of *M. balsamina* leaf extract on TGF- β levels in BCG-immunized rats

For further identification of immunomodulatory effect of *M. balsamina* extract, the levels of TGF- β were measured

as a representative of Th2 and anti-inflammatory cytokines. *M. balsamina* extract showed divergent effects on TGF- β levels depending on the dose. A significant increase ($p \leq 0.05$) of TGF- β levels was observed after 7 and 14 days of treatment with 50 and 100 mg/kg (Figure 5). In contrast, daily administration of 200 mg/kg of *M. balsamina* extract significantly ($p \leq 0.05$) reduced TGF- β levels in a time-dependent manner (Figure 6). *M. balsamina* leaf extract exhibited divergent effect on TGF- β levels.

Effects of *M. balsamina* leaf extract on IL-10 levels in BCG-immunized rats

The levels of IL-10 were measured after daily administration of three doses of *M. balsamina* extract. In

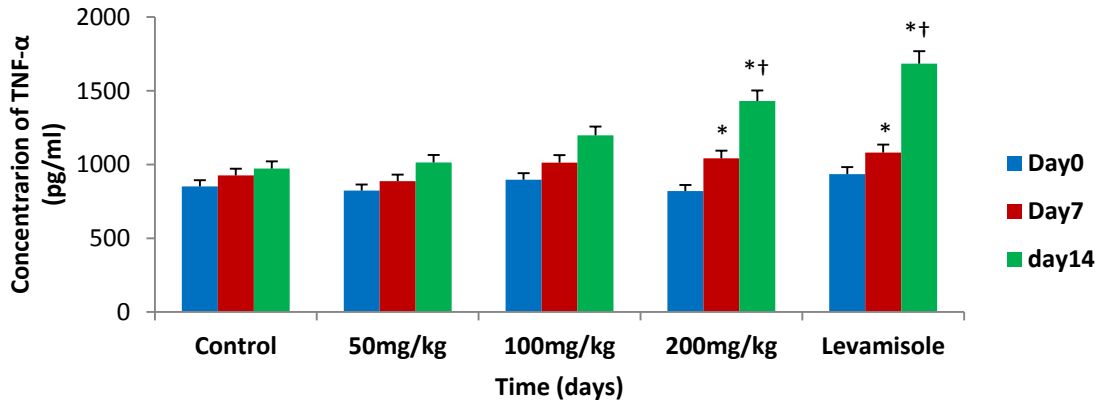


Figure 4. Time-dependent effects of *M. balsamina* on TNF- α levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. *, $p \leq 0.05$, versus day 0. †, $p \leq 0.05$ versus day 7.

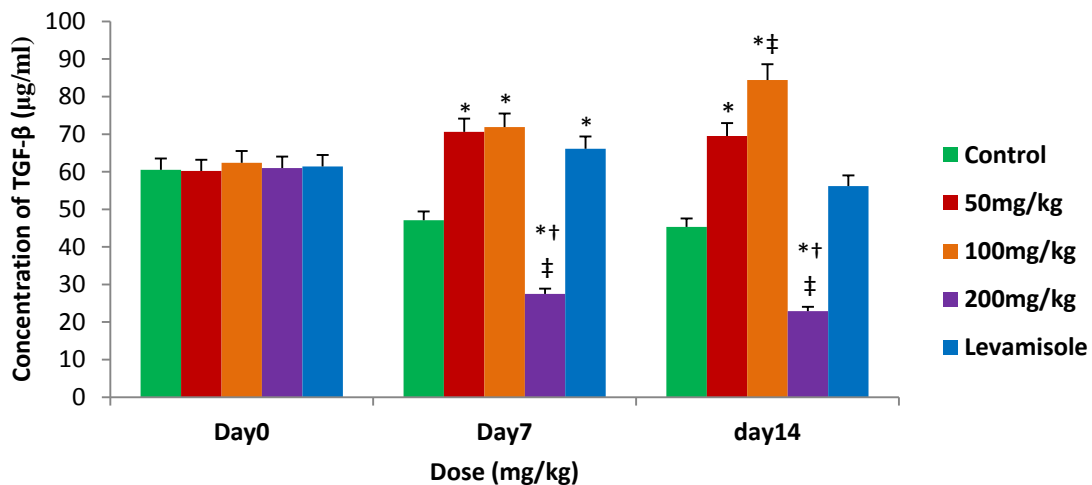


Figure 5. Dose-dependent effects of *M. balsamina* on TGF- β levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. *, $p \leq 0.05$, versus control. †, $p \leq 0.05$ versus 50 and 100 mg/kg. ‡, $p \leq 0.05$ versus levamisole.

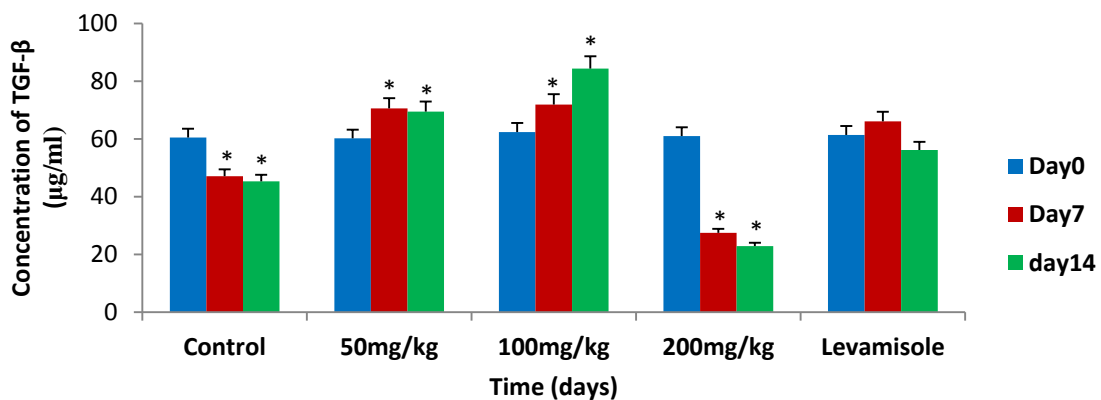


Figure 6. Time-dependent effects of *M. balsamina* on TGF- β levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D. *, $p \leq 0.05$ versus day 0.

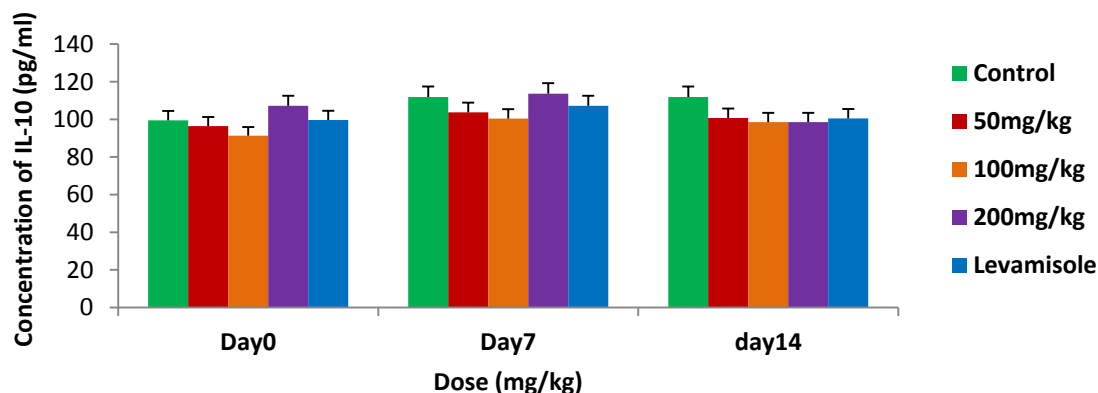


Figure 7. Dose-dependent effects of *M. balsamina* on IL-10 levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D.

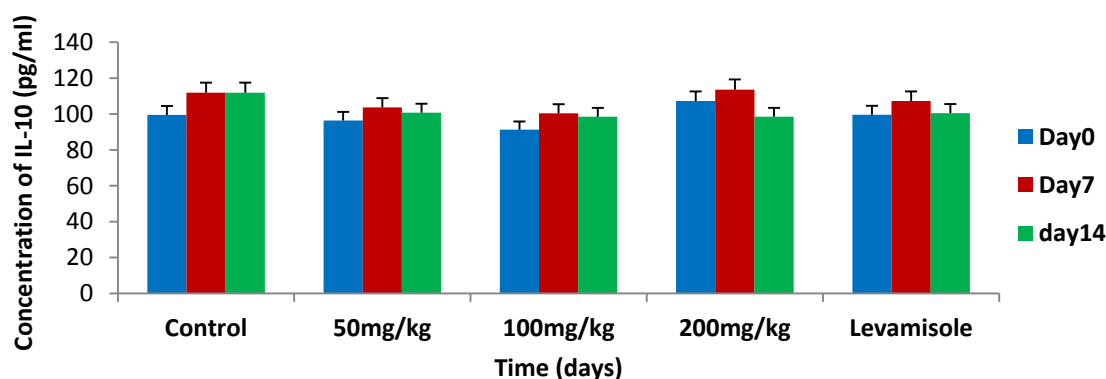


Figure 8. Time-dependent effects of *M. balsamina* on IL-10 levels. Rats were treated with *M. balsamina* extracts (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or only vehicle. Data are presented as mean \pm S.D.

this respect, neither dose-dependent nor time-dependent effects of *M. balsamina* extract on IL-10 levels were observed on the three administered doses (Figures 7 and 8). Therefore, no changes were observed on IL-10 levels after treatment with *M. balsamina* extract.

DISCUSSION

Considerable attention has been paid to plant-derived products such as alkaloids, flavonoids, terpenoids, and polysaccharides due to their diverse pharmacological properties including anti-inflammatory, hepatoprotective, anti-diabetic, antimicrobial, antiviral, cytotoxic and immunomodulatory effects (Abou 2016; Kamarudin et al., 2017). Plant-based immunomodulators may act by stimulating both specific and non-specific immunity, and can provide an effective and safe alternative to conventional chemotherapy for a variety of immune disorders (Jantan et al., 2015).

This study aimed to determine the immunomodulatory

effects of *M. balsamina* that has been used traditionally to treat various infectious diseases (Madureira et al., 2012). The levels of IFN- γ , TNF- α , IL-10 and TGF- β cytokines were quantified using enzyme linked immunosorbant assay (ELISA). Selection of cytokines is based on their key role in innate and adaptive immunity which would be cell-mediated or antibody-mediated immunity. Indeed, the balance between Th1 cytokines (IFN- γ and TNF- α) and Th2 cytokines (IL-10 and TGF- β) is critical for directing the immune response toward cell-mediated or humoral-mediated responses. Thus, any factors interfere with Th1/Th2 axis might affect the outcome of the immune response (Lucey et al., 1996).

In this study, *M. balsamina* showed a significant stimulation of Th1 response indicated by the significant increase of IFN- γ and TNF- α cytokines which are known to be produced by Th1 CD⁴⁺ T cells and lesser quantity is produced by CD⁸⁺ T cells (Abbas et al., 2014). The significant elevation of both IFN- γ and TNF- α cytokines produced by higher doses indicates the immunostimulatory effect of *M. balsamina* which requires

daily administration of the plant extract for 14 days or more. Thus TNF- α and IFN- γ increment at the higher doses reflects activation of Th1 response and cell-mediated immunity that is effective in eliminating virus-infected cells and participates in defense against fungi, protozoan, cancers, and intracellular bacteria (Abbas et al., 2014).

The immunostimulatory effects of other *Momordica* species have been coincidentally described by a number of *in vitro* and *in vivo* studies. Leafy stem juices of *M. charantia* induced significant T cell proliferation and enhanced IFN- γ production (Fachinan et al., 2017). Augmented cell-mediated and antibody-mediated immunity evident by increased Delayed Type Hypersensitivity (DTH) and Haemagglutinating Antibody Titre (HAT), respectively, was observed after oral administration of *Momordica charantia* to immunized rats (Prasad et al., 2010). Moreover, the immune-promoting properties of *M. charantia* were demonstrated by enhancing natural killer cells level, IL23a and IL1 β expression in cancer patients (Bhattacharya et al., 2017; Rao, 2018). Polysaccharide of *M. charantia* significantly increased the carbolic particle clearance index, spleen index, thymus index and NK cell cytotoxicity to normal control levels in immunosuppressed mice (Deng et al., 2014). *Momordica cochinchinensis* proliferated different cells of the immune system including splenocytes, splenic lymphocytes and bone marrow cells, in a manner comparable to that of Concanavalin A (Tsoi et al., 2006). In support of the immune-enhancing properties of *M. cochinchinensis* and *M. momordica* saponins were tested as vaccine's adjuvant and reported to stimulate secretion of broad range of cytokines, suggesting that saponins may act by triggering innate immunity (Song and Hu, 2009). Additionally, saponin derived from *M. cochinchinensis* reduced the production of nitric oxide (NO), and may be considered a bioactive immunomodulator with anti-inflammatory properties (Yu et al., 2017). The immunomodulatory activity of *Momordica* species might be attributed to the presence of similar phytochemicals among these variable species. Therefore, the immunostimulatory effects of *M. balsamina*, due to enhancement of TNF- α and IFN- γ levels, may justify the usefulness of this plant as antiviral, anticancer and against numerous infectious diseases. Low doses (50 and 100 mg/kg) of *M. balsamina* extract increased TGF- β levels while the high dose (200 mg/kg) reduced the level of TGF- β . The obvious inhibitory effect of high dose (200 mg/kg) of *M. balsamina* extract on TGF- β levels might be attributed to the tolerance of T-regulatory cells (Treg cells), the main producers of TGF- β . This possibly is supported by elevated IFN- γ and TNF- α levels at dose 200 mg/kg. Therefore, it is logic to observe such an inhibitory effect on TGF- β levels upon IFN- γ induction (Goldstein et al., 2013). It was previously documented that the elevated TGF- β levels have been correlated with cancer and fibrosis severity; and

strategies to block TGF- β action have been developed to antagonize excessive TGF- β signaling activity in the aforementioned disorders (Walton et al., 2017; Xie et al., 2017). In this study, *M. balsamina* extract at high dose (200 mg/kg) increased TNF- α and IFN- γ levels and suppressed TGF- β levels. On the basis of these observations, *M. balsamina* may be a good candidate for cancer treatment at high doses (\geq 200 mg/kg). Increased TGF- β level observed at low doses (50 and 100 mg/kg) may contribute to anti-inflammatory and antioxidant effects of *Momordica* species (Nagarani et al., 2014).

The immunostimulatory activity of *M. balsamina* extract may be due to its phytoconstituents such as saponins, flavonoids, sterols and triterpenoids which were reported to modulate the immune system (Kumar et al., 2012; Venkatalakshmi et al., 2016; Nagarani et al., 2014).

Conclusion

M. balsamia poses immuno-enhancing properties by stimulating cell-mediated immune responses via elevation of IFN- γ and TNF- α levels. Moreover, low doses of the plant have anti-inflammatory activity through elevating TGF- β levels. Therefore the immunostimulatory and anti-inflammatory effects of *M. balsamina* can justify its antiviral and anticancer activities and activity against numerous infectious and inflammatory diseases. Therefore, *M. balsamia* may play a role in strengthen human immunity and improving health. Different parts of *M. balsamia* are rich sources of triterpenoids, carotenoids, saponins, flavonoids that may potentially be used as immunomodulators and antioxidants in nutraceutical industries.

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CONFLICT OF INTERESTS

No potential conflicts of interest were identified in this study.

ABBREVIATIONS

IL-10, Interleukin-10; **TGF- β** , Transforming growth factor-

beta; **TNF- α** , Tumor necrosis factor-alfa, **IFN- γ** , Interferon-gamma.

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Full Length Research Paper

Comparative study of proximate, mineral and phytochemical analysis of the leaves of *Ocimum gratissimum*, *Vernonia amygdalina* and *Moringa oleifera*

M. D. Olumide^{*}, O. A. Ajayi and O. E. Akinboye

Department of Agriculture, School of Science and Industrial Technology, Babcock University, Ilishan Remo, Ogun State, Nigeria.

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The aim of this study is to make a comparative analysis of the proximate, mineral and phytochemical compositions of the leaves of *Ocimum gratissimum*, *Vernonia amygdalina* and *Moringa Oleifera* plants found within our vicinity in Nigeria. The analyses were investigated in accordance with standard procedures and compared. Of the 3 plants, *M. oleifera* had highest ($P<0.05$) crude fat, crude protein and total ash but lowest in carbohydrate content, whereas, *V. amygdalina* with highest fiber had the lowest crude fat and total ash. Crude fiber and crude protein were lowest in *O. gratissimum*. Also, *O. gratissimum* showed the highest composition of calcium, potassium, copper and zinc content compared to *V. amygdalina* and *M. oleifera*. Phytate and saponin were significantly predominant ($P<0.05$) in *O. gratissimum* relative to *V. amygdalina* and *M. oleifera*, whereas flavonoid and tannin were highest ($P<0.05$) in *V. amygdalina*. However, *M. oleifera* had the highest content of alkaloids among the three plants. The results showed that three leaves evaluated contained varying amount of the proximate, minerals and phytochemicals, hence, the regular use of these leaves are recommended.

Key words: Proximate, minerals, phytochemical, phytate, saponin, tannin.

INTRODUCTION

In Africa, many studies have indicated that a vast number of indigenous plants play a significant role in the diet of the populace (Muhammed et al., 2011). Plants are the cheapest and most available sources of important nutrients, supplying the body with minerals, vitamins and some hormone precursors, protein, energy and essential amino acids (Amaechi, 2009). Medicinal plants are plants that contain substances that can be used for therapeutic purposes or for synthesis of drugs (Sofowora, 2008).

Most tropical countries are blessed with a diversity of foodstuffs which play a basic role in nutrition and healthy body development. Unfortunately, an estimate of 789 million people in developing countries still suffers from malnutrition, especially infants and children of rural areas (WHF, 2005). Malnutrition can be tremendously reduced with an increased use of foods rich in energy, proteins, iron and vitamin A most especially those from the rural environment. The lack of nutritional information and

^{*}Corresponding author. E-mail: olumidemartha@gmail.com, olumideM@babcock.edu.ng.

inadequate development of nutritionally improved products from local raw materials have direct bearing on nutrition. Much effort has been concentrated on seeds while leafy vegetables have largely been ignored. Recently in Africa, increased interest has been observed in the use of herbs to improve health; herbs could be regarded as one of the first real functional food, but has largely become forgotten food in the modern westernized diet. Culinary herbs are as important today as they were in ancient times for enhancing the flavour and taste of our foods as well as serving as a source of dietary medicine (Uhegbu et al., 2011). Ignorance concerning the nutritional properties and presence of some phytochemicals are the major reasons for under-utilization of these herbs. So many people consume vegetables because of their flavors and taste, and do not concern themselves with their nutritional composition (Oshodi, 1992; Ejoh et al., 1996).

Ocimum gratissimum (scent leaves) belongs to the family Lamiaceae and is found mostly in the tropical countries including; Nigeria, India, North and South America, Mexico and Brazil. It is a full developed flowering plant with roots, stem and leaves systems (Iwu, 1993). It prefers moist and fertile soils during growth, but can tolerate drought after flowering. It is naturally and traditionally used to relief pains and in the treatment of rheumatism, diarrhea, high fever, convulsions, diabetes, eczema, piles, skin infections, gastroenteritis, stomachache, cuts, wounds, inflammation, diuretic and as a repellent (Chitwood, 2003; Ilori, 1996).

Vernonia amygdalina (VA) is a shrub or small tree that grows throughout tropical Africa, and is popularly called bitter leaf because of its abundant bitter taste (Ekpo et al., 2007). The leaves contain a considerable amount of anti-nutritional factors like high level of tannic acid and saponin. Research has shown that *V. amygdalina* have some beneficial effect in disease management of poultry (Dakpogan, 2006) such as anti-bacterial and anti-parasitic and anti-oxidant (Erasto et al., 2009) and as growth promoter by enhancing the gastro intestinal enzymes thus increasing feed conversion efficiency (Olabatoke and Oloniruba, 2009). Beside that it is used as an indigenous vegetable in human nutrition, the plant has also acquired significant relevance in human medicine having been proven to possess potent anti-malarial as well as anti-tumorigenic properties (Izevbigie, 2003).

Moringa oleifera belongs to the Moringaceae family and is considered to have its origin in the north-west region of India, south of the Himalayan Mountains. It is now widely cultivated and has become naturalized in many locations in the tropics (Fahey et al., 2001). It is a rapidly-growing tree also known as horseradish tree or drumstick tree. All parts of the Moringa tree are edible and have long been consumed by humans. Recently, there has been interest in the utilization of Moringa as a protein source for livestock (Sarwatt et al., 2002). Furthermore, there is the

possibility of obtaining large amount of high quality forage from Moringa without expensive inputs due to favorable soil and climatic conditions for its growth. Sarwatt et al. (2004) reported that Moringa foliage's are a potential inexpensive protein source for livestock feeding. The advantages of using Moringa for a protein resource are numerous, and include the fact that it is a perennial plant that can be harvested several times in one growing season and also has the potential to reduce feed cost.

Despite various reports available on medicinal plants, detailed comparative nutritional information on these three leaves is not available. However, to understand the roles played in human and animal nutrition, knowledge of proximate, mineral and phytochemical composition is fundamental to the understanding of the mode of action of these medicinal plants in general. This will be useful for the nutritional education of the public as a means to improve the nutritional status of the population. Therefore, this study quantitatively analyzed the proximate, mineral and phytochemical compositions of the leaves of *O. gratissimum*, *V. amygdalina* and *M. oleifera* that are commonly found within our vicinity.

MATERIALS AND METHODS

Leaf meal preparation

Mature fresh leaves of *O. gratissimum*, *V. amygdalina* and *M. oleifera* were identified by the Agronomy Department and harvested around the Teaching and Research Farm of Department of Agriculture and Industrial Technology, Babcock University, Ilishan-Remo, Ogun State. Harvesting was done between the hours of 16:00 and 17:00 when the plants must have completed their light stage of photosynthetic process for the day. The quantity of leaves needed was air dried at an average room temperature of 27°C for seven days and then milled with a hammer mill sieve of 0.02 mm pore size to obtain a fine powdery dust. The powdered test samples were stored in a dry, clean container with lid for further analysis.

Chemical analysis

Proximate analysis

The moisture content was determined by drying at 105°C in an oven until a constant weight was reached. For total ash determination, the leaves samples were weighed and converted to dry ash in a muffle furnace at 450 and at 550°C for incineration. The crude fat content was determined by extraction with hexane, using a Soxhlet apparatus. All these determinations were carried out according to AOAC (1990). Kjeldahl method was used for crude protein determination. Carbohydrate content was determined by calculating the difference between the sums of all the proximate compositions from 100%. Energy values were obtained by multiplying the carbohydrate, protein and fat by the Atwater conversion factors of 17, 17 and 37, respectively (Kilgour, 1987).

Minerals analysis

Mineral analyses were carried out according to Martin-Prevel et al. (1984). Elemental analyses were carried out using an atomic absorption spectrophotometer and flame photometer to determine calcium, sodium, potassium and magnesium content. Iron, copper

Table 1. Proximate composition of the leaves of *O. gratissimum*, *V. amygdalina* and *M. Oleifera*.

Parameter	Percentage composition (%) of leaves			SEM
	<i>Ocinum gratissimum</i>	<i>Vernonia amygdalina</i>	<i>Moringa oleifera</i>	
Moisture	13.60	14.50	14.20	1.15
Crude fat	4.20 ^b	3.60 ^c	4.60 ^a	0.61
Crude protein	14.35 ^c	21.00 ^b	25.90 ^a	2.29
Total ash	10.50 ^b	7.10 ^c	15.60 ^a	1.83
Crude fiber	7.60 ^c	8.90 ^a	8.00 ^b	0.01
Carbohydrate	49.75 ^a	44.90 ^b	31.70 ^c	7.80

Means with similar alphabets along the rows were not statistically different ($p \leq 0.05$) level of probability.

Table 2. Mineral composition of leaves of *O. gratissimum*, *V. amygdalina* and *M. oleifera*.

Parameter	Percentage composition (%) of leaves			SEM
	<i>Ocinum gratissimum</i>	<i>Vernonia amygdalina</i>	<i>Moringa oleifera</i>	
Calcium	1.92 ^a	1.27 ^b	1.25 ^b	0.11
Magnesium	0.07	0.06	0.06	0.01
Potassium	4.30 ^a	3.70 ^b	2.25 ^c	0.30
Sodium	0.16	0.12	0.14	0.01
Iron	1.52 ^b	2.48 ^a	1.55 ^b	0.41
Copper (mg/g)	17.60 ^a	16.55 ^b	3.90 ^c	0.01
Zinc (mg/g)	57.50 ^a	45.80 ^b	23.40 ^c	4.98

and zinc were determined calorimetrically. The concentration of each element in the leaf sample was calculated on a dry matter basis.

Phytochemical analysis

Phytochemical analysis was conducted to determine the presence of phytate, saponin, flavanoid, tannin and alkaloid while the quantification of saponin was done by afrosimetric method (Koziol, 1991). The gravimetric method (Haborne, 1993) was used in determination of alkaloid and flavonoid contents. All the analyses were done using triplicate samples.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using Statistical Package for Social Sciences version 17.0 for windows, SPSS Inc. Means were separated using Duncan Multiple Range Test where significant.

RESULTS

Proximate composition

The proximate composition of the leaves of *Ocinum gratissimum*, *V. amygdalina* and *M. oleifera* presented in Table 1 showed that the moisture content value of the leaves were not significantly different but ranged from 13.60 – 14.50%. The highest moisture content value was obtained from *amygdalina* (14.50%) while the least value

was obtained from *O.gratissimum* leaves.

Of the three leaves, *M. oleifera* had the highest crude fat (4.60%), crude protein (25.90%) and total ash (15.60%) content but lowest carbohydrate composition (31.70%); whereas, *V. amygdalina* with the highest crude fiber (8.90%) had the lowest crude fat (3.60%) and total ash (10.71%). *O. gratissimum* demonstrated the lowest content of crude protein and crude fiber (14.35 and 7.60% respectively).

Mineral composition

The result of mineral composition of the leaves of *Ocinum gratissimum*, *V. amygdalina* and *M. oleifera* were shown in Table 2. The results showed that *O. gratissimum* was significantly highest ($P < 0.05$) in calcium, potassium, copper and zinc (1.92%, 4.30%, 17.60, 57.50 mg/g respectively) content compared to the other two plant leaves. However, *V. amygdalina* was significantly highest in iron (2.84%) while the lowest values of potassium (2.25%), copper (3.90 mg/g) and zinc (23.40 mg/g) were obtained from *M. oleifera* leaves. The magnesium and sodium contents were similar in the three leaves and the value ranged from 0.06 – 0.07% and 0.12 – 0.16% respectively. Iron values obtained ranged from 1.52 - 2.48%. The lowest value was obtained from *O. gratissimum* while the highest value was obtained from *V. amygdalina* leaves. The three leaves were significantly

Table 3. Phytochemicals of the leaves of *O. gratissimum*, *V. amygdalina* and *M. oleifera*.

Parameter	Percentage composition (%) of leaves			
	<i>Ocinum gratissimum</i>	<i>Vernonia amygdalina</i>	<i>Moringa oleifera</i>	SEM
Phytate	10.44 ^a	7.84 ^c	9.05 ^b	0.01
Saponin	1.10 ^a	0.60 ^b	0.40 ^b	0.15
Flavanoid	25.30 ^b	30.45 ^a	26.15 ^b	1.01
Tannin	0.03 ^b	0.17 ^a	0.02 ^b	0.00
Alkaloid	2.18 ^b	2.04 ^c	2.80 ^a	0.01

high in zinc. The value ranged from 23.40 – 57.50 mg/g. The lowest value was obtained from *M. oleifera* and the highest value from *O. gratissimum* leaves.

Phytochemical

The phytochemical analysis as shown in Table 3 revealed that phytate, saponin, flavanoid, tannin and alkaloid were present in the leaves. The phytate and saponin were significantly predominant ($P>0.05$) in *O. gratissimum* (10.44 and 1.10%), relative to *V. amygdalina* and *M. oleifera*, whereas flavanoid (25.30%) and tannin (0.03%) were highest ($P<0.05$) in *V. amygdalina* (30.24 and 0.17%). However, *M. oleifera* had the highest content of alkaloid among the three plants.

DISCUSSION

Proximate composition

The moisture content recorded in this study is significantly higher than the value obtained for *O. gratissimum* (6.67%) and (7.90%) for *V. amygdalina* leaves by Belewu et al. (2009) but lower than the value (20.08%) obtained by Ejoh et al. (2007). The moisture content value obtained for Moringa in this study (14.20%) was however higher than (5.90%) obtained by Yameogo et al. (2011) for *M. oleifera* leaves. The low level of moisture in all the samples investigated suggests that the leafy vegetables would store for long without spoilage since a higher water activity could enhance microbial action bringing about spoilage. In this study, the air dried leaves low moisture contents will favour their preventive properties against microbial attacked and thus the storage life of the air-dried leaves will be high. Although, these values were found to be low when compared to the 24.0% recorded for *A. senegalensis* (Yameogo et al., 2011). The fat content was also comparably low to the values obtained by Belewu et al. (2009) for *O. gratissimum* (11.75%) and *V. amygdalina* (13.40%), and the value reported by Ejoh et al. (2007) for *V. amygdalina* (4.70%). The low fat content of the samples studied suggests that the plant cannot serve as oil vegetables but

may be useful for individuals on weight-reducing diets (Emebu and Anyika, 2011).

The high protein content values recorded for both *V. amygdalina* and *M. oleifera* leaves were found to be similar to that reported by Belewu et al. (2009) for *O. gratissimum* (20.78%) and *V. amygdalina* (28.88%) but higher than the value 19.23% obtained by Ejoh et al. (2007). The crude protein obtained in this study for Moringa (25.90%) was however lower than that (27.50%) obtained by Sun et al. (2018). The high protein values recorded for *V. amygdalina* and *M. oleifera* in this study suggest that the leaves can be ranked as a potential source of plant protein and therefore be used as a protein supplement in the diet.

The total ash values obtained for *O. gratissimum* and *V. amygdalina* were favorably compared to the range of 5.43 - 5.75% reported for some edible woody plants (Emmanuel et al., 2011) and higher than the range of 0.38 - 1.9% for selected vegetables grown in Peshawar (Bangash et al., 2011). The values obtained in this study were also higher than that obtained by Belewu et al. (2009) for *O. gratissimum* (3.58%) and *V. amygdalina* (4.85%). The values of ash observed in all the leaves is an indicator that these samples are good sources of minerals when compared to the values obtained for cereals (FAO, 1980).

The present study reports crude fiber values that are slightly lower than values reported by Ejoh et al. (2007) and Belewu et al. (2009) for *V. amygdalina* and *O. gratissimum* leaves. Also, the value obtained in this study for *M. oleifera* (8.00%) was lower than the value (9.40%) obtained by Sun et al. (2018). The differences in values obtained could be due to geographical location or soil type. This indicates that these leaves can be included in the diets without any deleterious effects; however, the carbohydrate content of *M. oleifera* leaves obtained in this study was lower (31.70) compared to that obtained (54.61) by Mónica et al. (2015). The value obtained for carbohydrate in the leaves of *V. amygdalina* was similar to that obtained by Okeke et al. (2015).

Total carbohydrate levels in these leaves were relatively high except that of Moringa. These carbohydrate sources are not generally used because most of them remain undigested. The difference observed may be due to the physiological state of the plant before harvesting.

(Singhal and Kulkarni, 2007).

Mineral

The calcium values obtained in this study are relatively high, *O. gratissimum* had the highest level of calcium, while that of *V. amygdalina* and *M. oleifera* were not significantly different. Calcium is necessary for the strong bones and teeth. This indicates that the three leaves can provide part of the daily required calcium in the body when consumed. Magnesium and sodium percentages obtained in this study were not comparatively different among the leaves. The potassium content of the leaves of *O. gratissimum*, *V. amygdalina* and *M. oleifera* differ significantly in values, with the highest obtained from *O. gratissimum* (4.30) and the lowest was obtained from the leaves of *M. oleifera* (2.25). Potassium is responsible for nerve action and some osmo-regulation in the body fluid. (Odoemena and Ekanem, 2006). The values of calcium, magnesium, sodium and iron content fell within the range of 0.05 - 0.53% and 0.04% reported for maize and millet grains (Brou et al., 2009) and *Hymenocardia ulmoides* and *V. ferruginea* leaves (Andzouana and Mombouli, 2011), respectively. The iron content of the tested leaves were significantly different with the highest value obtained from *V. amygdalina* (2.48) , followed by that of *Moringa* (1.55) which was not significantly different from the value obtained from *O. gratissimum* (1.52). Iron is an essential trace element for hemoglobin formation, normal functioning of central nervous system and in oxidation of carbohydrates, protein and fats. (Adeleye and Otokiti, 1999). This indicates that *V. amygdalina* can contribute this mineral and enhance their availability in daily life. The values of copper in the leaves of *Ocimum*, *Vernonia* and *Moringa* varied from 3.90 -17.60 mg/g. Copper deficiency has been reported to cause cardiovascular disorders as well as anaemia. Significant difference was also observed in the values of zinc obtained for the three leaves with the least value obtained from *Moringa* (23.40 mg/g), while the highest value was obtained from the leaves of *O. gratissimum* (57.50 mg/g).

Phytochemicals

The analysis of the three leaves revealed that they contained appreciable amount of phytochemical. The presence of saponin in the samples agreed with the results reported by Edeoga et al. (2006). Saponin is used in medicine and pharmaceutical industries because of its foaming ability with frothy effect. Flavanoid were strongly present in all the leaves, the values obtained were significantly different. The highest value were obtained from the leaves of *V. amygdalina* (30.45%) followed by the *M. oleifera* (26.15) and the least value from *O. gratissimum* (25.30) leaves. The presence of flavonoid in

the leaves supported the report of Edeoga et al. (2006); flavonoids are well known antioxidants (Tiwari and Rao, 2002). Although tannins are present in the tested leaves in minute levels, *V. amygdalina* had the highest percentage (0.17) while the lowest value was obtained from the leaves of *M. oleifera* (0.01). This is in agreement with the findings of Belewu et al. (2009), and also in line with the previous reports of Tiwari and Rao (2002) who reported high level of tannin and saponin for *V. amygdalina* which was responsible for the bitter taste of the plant. Tannins are known to have antiviral, antibacterial, antitumor and antidiabetic properties via carbohydrate modulation on the GIT due to the presence of these phytochemicals (Bnouham et al., 2006). The presences of tannins are responsible for the astringent flavour in these leaves. Alkaloids values obtained in this study were significantly different. The lowest values were obtained from the leaves of *V. amygdalina* (2.04%) followed by *O. gratissimum* (2.18%), while the highest values were obtained from the leaves of *M. oleifera* (2.80%). The presence of alkaloids in these leaves samples supported the reports of Edeoga et al. (2006). Alkaloids are known to play some metabolic roles. The compounds have protective role in animal and its constituent of most valuable drugs.

Conclusion

The study revealed that all the leaves are good sources of crude proteins, crude fat and minerals with levels particularly higher in most cases in *M. oleifera*, followed by *O. gratissimum* and the least by *V. amygdalina*. *O. gratissimum* had the highest level of calcium and potassium followed by *Moringa* but *V. amygdalina* has a higher level of iron. These leaves also contain high levels of some antinutritional factors. *V. amygdalina* had highest level of flavanoid and tannin, while phytate and saponin were higher in *O. gratissimum*. In conclusion, these commonly available plants leaves are good sources and potent bioactive compounds which could be used for therapeutic purpose or as precursors of synthetic drugs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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