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RESEARCH ARTICLE

Glycosides Isolated from Leaf Extract of *Phyllanthus muellerianus* (Kuntze) Excell (Phyllanthaceae) Upregulated Cell-mediated Innate Immunity

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Glycosides Isolated from Leaf Extract of Phyllanthus muellerianus (Kuntze) Excell (Phyllanthaceae) Upregulated Cell-mediated Innate Immunity

SUMMARY

Intracellular pathogens are mainly eliminated by cell-mediated immunity from phagocytic cells like neutrophils, macrophages, and monocytes. Tannin glycosides, 1-O-galloyl-6-O-luteoyl-α-D-glucopyranoside (1) and 3-O-methylellagic acid 3'-O-α-rhamnopyranoside (2) were isolated from the ethyl acetate fraction (EF) of the methanol leaf extract of Phyllanthus muellerianus. Structures of the isolated compounds were elucidated by 1H-NMR and by mass spectroscopy. Effects of the isolated compounds on the phagocytic competence of macrophages and neutrophils using in vitro models were evaluated. Tannin glycosides 1 and 2 significantly ($p<0.05$) increased both phagocytic ability and intracellular killing capacity of neutrophils. Present study established that tannin glycosides stimulated cell-mediated innate immunity by increasing the phagocytic function of neutrophils and thus may be helpful to both the clinical and prophylactic treatment of intracellular microbial infections.

Key Words: *Glycosides, cell-mediated immunity, phagocytosis, neutrophils, macrophages.*

Phyllanthus muellerianus (Kuntze) Excell (Phyllanthaceae) Yaprak Ekstresinden İzole Edilen Glikozitlerin Hücre-Aracılı Doğuştan Bağışıklığı Yukarı Regüle Etmesi

ÖZ

Hücre içi patojenler esas olarak nötrofiller, makrofajlar ve monositler gibi fagositik hücreler tarafından sunulan hücre aracılı bağışıklık tarafından elimine edilir. İki adet tanen glikozit, 1-O-galloil-6-O-luteoil-α-D-glukopiranozit (1) ve 3-O-metilelajik asit 3'-O-α-ramnopiranozit (2) Phyllanthus muellerianus yaprak metanol ekstresinin etil asetat fraksiyonundan (EF) izole edilmiştir. İzole edilen bileşiklerin yapıları, 1H-NMR ve kütle spektroskopisi ile aydınlatılmıştır. İzole edilen bileşiklerin makrofajların ve nötrofillerin fagositik yeterliliği üzerindeki etkileri in vitro modeller kullanılarak değerlendirilmiştir. Bileşik 1 ve 2, nötrofillerin hem fagositik kabiliyetini hem de hücre içi öldürme kapasitesini önemli ölçüde ($p<0.05$) arttırmıştır. Bu çalışma, tanen glikozitlerin, nötrofillerin fagositik fonksiyonunu artırarak hücre aracılı doğuştan gelen bağışıklığı uyardığını ve bu nedenle hücre içi mikrobiyal enfeksiyonların hem klinik hem de profilaktik tedavisinde yararlı olabileceğini ortaya koymuştur.

Anahtar Kelimeler: *Glikozitler, hücre-aracılı bağışıklık, fagositoz, nötrofiller, makrofajlar.*

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INTRODUCTION

The immune system is the body's natural defense against foreign substances. It is composed of biological structures, a network of cells and processes within the body that works to protect it against myriads of pathogens (Froy et al. 2007). An immune system must detect various agents, including viruses, bacteria, fungi, parasitic worms and particulate materials, and distinguish them from the body's healthy tissues. The body then mounts a counter-attack against these infectious agents and thus wards off their harmful effects. Main line of defense in the innate immune response is the recruitment of phagocytic cells (neutrophils, monocytes, macrophages, etc.) to the site of infection (cell-mediated innate immunity). Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer, and infectious diseases (Patwardhan et al. 1991). Degree to which a patient becomes abnormally susceptible to infections by microbial agents depends on the extent of immunosuppression. Suppression of the immune system is characterized by impairment of phagocytosis. This results from a reduction in the number and phagocytic function of the neutrophils and macrophages, as well as impairment of the intracellular killing capacity of these cells.

Immunosuppression predisposes to attack by infectious agents and allows opportunistic pathogens to overwhelm the host to cause secondary infections (Rao et al. 1994). Phagocytosis is the primary defense mechanism against foreign body invasion, and it is offered by neutrophils and macrophages. Compounds with immunomodulatory activities are replete with many adverse effects. Microbial resistance to almost all the available chemotherapeutic agents has shifted research interest to explore the enhancement of immune response against infectious agents using natural products. *In vitro* and *in vivo* tests are available for screening of phagocytic competence of immune cells. Medicinal plants derived compounds have been reported to modulate phagocytic functions (Fujinaga et al, 2000; Byrd-Leifer et al. 2001; Gertsch et al. 2004; Schepetkin et al. 2009).

Phyllanthus muellerianus (Kuntze) Exell (Phyllanthaceae) is an evergreen shrub with numerous stems, distributed in tropical and subtropical regions of West Africa (Boakye-Gyasi et al. 2016). Different parts of *P. muellerianus* are used in folkloric medicine to treat dysmenorrhea, fevers, convulsions, toothache, anemia, dysentery, diarrhea, and constipation (Ben-Bala, 2008; Nisar et al, 2018). Immunomodulatory effects of *P. muellerianus* on humoral and cellular adaptive immunity have been reported (Ofokansi et al. 2018). The present study aimed to investigate the cell-mediated innate immunity potentials of *Phyllanthus muellerianus* by evaluating the phagocytic competence of the isolated compounds using *in vitro* models.

MATERIAL AND METHODS

Instrumentation

^{1}H-NMR spectra were recorded using Bruker ARX 300 NMR spectrometer with chemical shifts given in ppm (δ) using CD_3OD and coupling constants (J) in Hz. HPLC-ESIMS measurements were recorded on a Thermo Finnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system that included an online photodiode array detector. Analytical HPLC analysis was performed with a Dionex HPLC system (Column: 125 *x* 4 mm, adsorbent: 5 µm Eurosphere 100 C18) with a photodiode array detector, employing a gradient of methanol in acidic water (pH = 2, 0.1% formic acid) from 5% to 100% in 35 min (Table 1). Detection was set at wavelengths 235, 254, 280 and 340 nm. semi-preparative HPLC separation was performed on a C18 column (300 *x* 8 mm, Eurosphere 100–10) on a Hitachi system equipped with a UV Detector and Flatbed Recorder and a flow rate of 5.0 mL/min. Vacuum liquid chromatography (VLC) purification was done in a glass column (30 *x* 3 cm, ID) using silica gel (230–400 mesh) as the adsorbent.

Table 1. Gradient for standard HPLC Analysis

Time (minutes)	Acidic water (%)	MeOH (%)
0	90	10
5	90	10
35	0	100
45	0	100
50	0	10
60	0	10

Chemicals and reagents

Methanol, *n*-hexane, ethyl acetate, lipopolysaccharide (LPS), and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich (Germany). Also used are Tween 80 (Janssen, Belgium), phosphate buffer saline, (PBS 8 g NaCl, 0.2 g KC1, 1.44 g Na_2PO_4, 0.24 g $KHPO_4$ in 1L distilled water, pH=7.4). Ethylenediaminetetraacetic acid (EDTA), silica gel 60, 70-230 mesh (Merck, Germany), Leishman, and carbofushsin stains (Hopskin and William, England). Nylon fibers were locally sourced. Neubauer counter (Gallenkamp), Indian ink were used. McFarland point five turbidity standards were prepared from barium chloride, tetraoxosulphate (VI) acid, and water.

Plant Material

Fresh leaves of *P. muellerianus* were collected in November 2018 from Uzo-Uwani Local Government Area of Enugu State, Nigeria. They were identified and authenticated by Mr. A. O. Ozioko of the International Centre for Ethnomedicine and Drug Development (InterCEDD) with a voucher specimen with number INTERCEDD062014. Leaves were air-dried for fourteen days and pulverized.

Extraction and Isolation Procedure

Powdered leaves (1.0 kg) were extracted with methanol using a Soxhlet apparatus. Methanol extract was concentrated *in vacuo* to obtain the dry extract (ME, 170 g). Extract (10.0 g) was fractionated using a glass column (150 *x* 1.5 cm, ID) on silica gel (70-230 mm mesh) and eluted successfully with *n*-hexane, ethyl acetate, and methanol to obtain the solvent fractions *n*-hexane fraction (HF: 0.25 g), ethyl acetate fraction (EF, 3.22 g) and methanol fraction (MF, 5.7 g). EF (3.0 g) was further purified by vacuum liquid chromatography using silica gel (230–400 mesh) as the stationary phase with a glass column (30 *x* 3 cm, ID) and eluted with a gradient of *n*-hexane in ethyl acetate (10:0, 8:2, 6:4, 4:6, 2:8, 0:10 each 500 mL) and of dichloromethane in methanol (9:1, 7:3, 5:5, 3:7,1:9, 0:10 each 1000 mL) to afford eleven sub-fractions (EF1-EF11) (Agbo et al. 2017). EF6 (135.6 mg) and EF7 (261.7 mg) were purified using semi-preparative HPLC with methanol-water as the mobile phase to yield compounds **1** (45.0 mg) and **2** (120 mg), respectively.

In vitro phagocytosis of killed Candida albicans

Preparation of Candida albicans Suspension

Candida albicans culture was incubated in Sabouraud dextrose broth overnight and centrifuged to form a cell button. Supernatant was discarded, and the cell button was washed 3-4 times with sterile PBS and then centrifuged. The washed cell button was resuspended in a mixture of PBS and rat serum (4:1). The *C. albicans* count was adjusted to 10^8 cells/mL using a 0.5 McFarland standard.

Slide preparation

A suspension of leukocytes was prepared from the buffy coat after centrifugation of rat blood collected in an EDTA container. Leukocyte suspension was adjusted to a concentration of 6.5 *x* 10^3 µL by diluting with PBS, and 0.2 mL of the rest was introduced into four sets of six test tubes arranged serially. Two-fold serial dilution of compounds 1 and 2 (100, 50, 25, and 12.5 µg/mL) was made. 0.1 mL of each concentration was introduced into the first four test tubes. The fifth and the sixth tubes contained PBS (0.1 mL) and a suspension of 100 µg LPS (0.1 mL) and served as negative and positive controls, respectively. Lines were incubated for 15 mins and, a 0.2 mL aliquot of the prepared *C. albicans* suspension was added into the test tubes. Lines were incubated for one hour, centrifuged at 500 rpm for 5 mins, and supernatant discarded. The cells were resuspended in 0.1 mL of PBS and a thin

film made with a drop on a clean glass slide. Smeared glass slide was dipped in methanol and stained with Giemsa thrice (Ganachari et al. 2004).

Phagocytosis evaluation

The number of *C. albicans* cells phagocytosed by Polymorphonuclear leucocytes (PMNs) on the slide was determined microscopically for 100 granulocytes using morphological criteria. Number of cells phagocytosed was taken as Phagocytic Index (PI) and compared with the basal PI of the control. Immunostimulant percentage (%) is calculated using the following equation (Ganachari et al. 2004):

$$Stimlation(\%) = \frac{PI_{test} - PI_{comtrol}}{PI_{control}} \times 100$$

Intracellular killing capacity of neutrophils

A suspension of leukocytes was prepared from the buffy coat after centrifugation of sheep blood collected in an EDTA container. Leukocyte suspensions were pooled and adjusted to a concentration of 17.0 *x* 10^3/μL by diluting with PBS. 0.5 mL of the rest was introduced into four sets of six test tubes arranged serially. Twofold serial dilution (100, 50, 25, and 12.5 μg/mL) of compounds **1** and **2** were made, and 0.1 mL of each concentration was introduced into the first four test tubes. The fifth and the sixth tubes contained PBS (0.1 mL) and a suspension of LPS (0.1 mL) and served as negative and positive controls, respectively. 0.2 mL aliquot of a freshly made 0.2% Nitroblue tetrazolium (NBT) solution was added to each tube and incubated at 37°C for 20 min. Lines were centrifuged at 500 rpm for 5 min and the supernatant was discarded. Cells were resuspended in 0.1 mL of PBS and, a thin film made. Film was dried, fixed by heating, and counterstained with dilute carbol-fuchsin for 15 sec. Slide was washed under tap water, dried, and viewed (100 *x*) under oil immersion. Two hundred neutrophils were counted, and proportion of NBT-positive cells containing blue granules determined. Procedure was repeated three times (Wilkinson 1981, Ganachari et al. 2004).

Statistical analysis

Results are expressed as mean ± SEM (n=3). Data obtained were analyzed using a one-way analysis of variance followed post hoc test (LSD) for multiple comparisons. Differences between mean observations were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The isolated compounds (Figure 1)

Properties of 1-O-galloyl-6-O-luteoyl-α-D-glucopyranoside (**1**)

Pale brown powder; retention time (t_R) of 18.023 mins; UV (MeOH) λ_{max} 222.0, 268.0 nm; ^{1}H (500 MHz CD_3OD) NMR data: See Table 2; Figures 2a & 2b; 3a & 3b. HPLC-MS *m/z* 657.0 $[M + Na]^+$ ($C_{27}H_{22}O_{18}$ + Na), 465 $[M-170]^+$ (loss of gallic acid), 303 $[M-170-162]^+$ (loss of hexose sugar).

Table 2. ^{1}H-NMR Data of Compound 1

	Compound **1**	
Position	δ_H^* (J in Hz)	$\delta_H^{a,**}$ (J in Hz)
1	6.36, d (2.16)	6.37, d (3.7)
2	3.98, d (2.0)	3.99, m
3	4.80, d (3.1)	4.81, m
4	4.46, m	4.47
5	4.51, t (9.4)	4.53, ddd (2.1, 5.0, 11.4)
6	4.96, m	4.99, dd (2.1, 11.1)
	4.16, dd (8.0, 11.0)	4.15, dd (5.0, 11.1)
2′	7.05, s	7.06, s
6′	7.05, s	7.06, s
5′′	6.66, s	6.66, s
5′′′	6.69, s	6.69
*Measured in (500 MHz), **Measured in MeOH-d_4 (500 MHz), a) (Okoye *et al.*, 2015)		

Properties of 3-O-methylellagic acid 3'-O-α-L-rhamnopyronoside (**2**)

Pale yellow powder; retention time (t_R) of 26.170 mins; UV (MeOH) λ_{max} 250.0, 364.0 nm. HPLC-MS *m/z* 484.9 $[M + Na]^+$ ($C_{21}H_{17}O_{12}$ + Na), 317.2 $[M-145]^+$ (loss of rhamnose) and 315 $[M-1-146]^-$ (loss of aglycone).

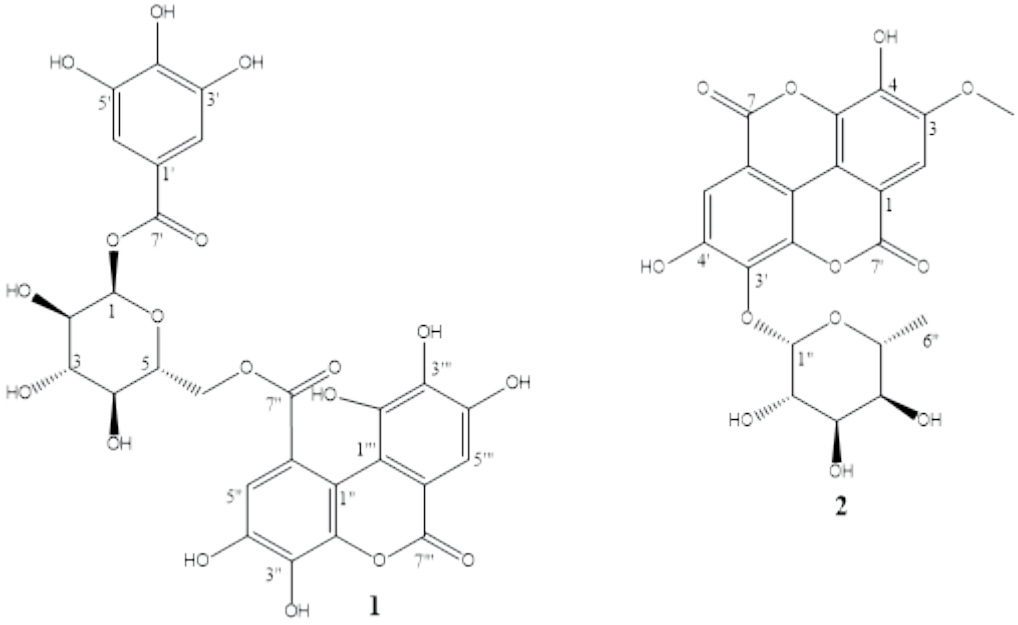

Figure 1: Structures of the Isolated Flavonoid glycosides

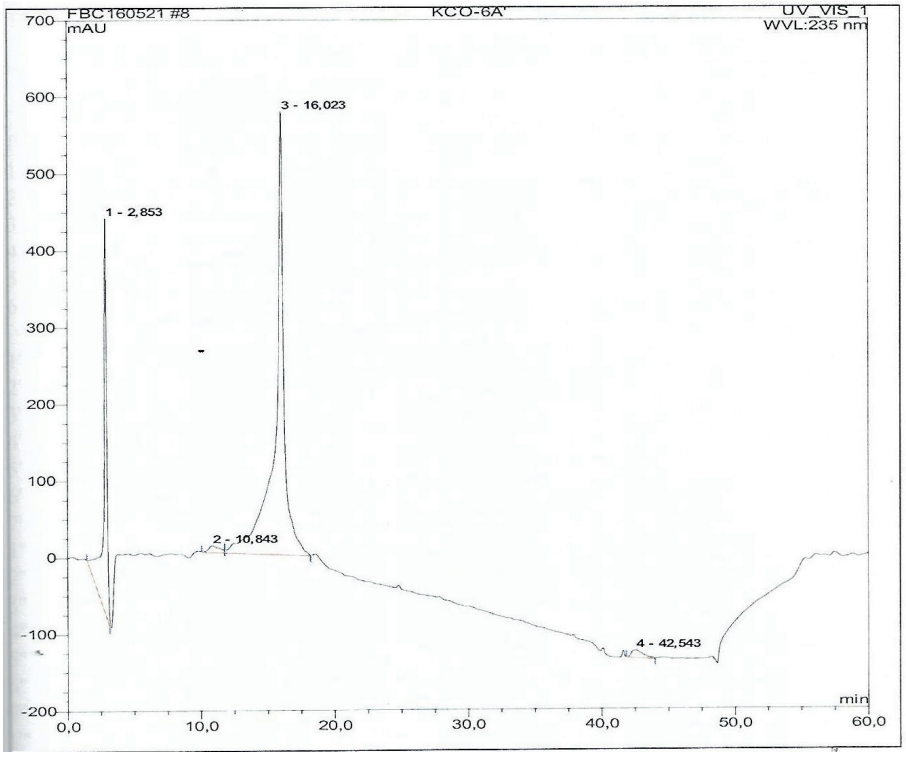

Figure 2a: HPLC Chromatogram of Compound 1

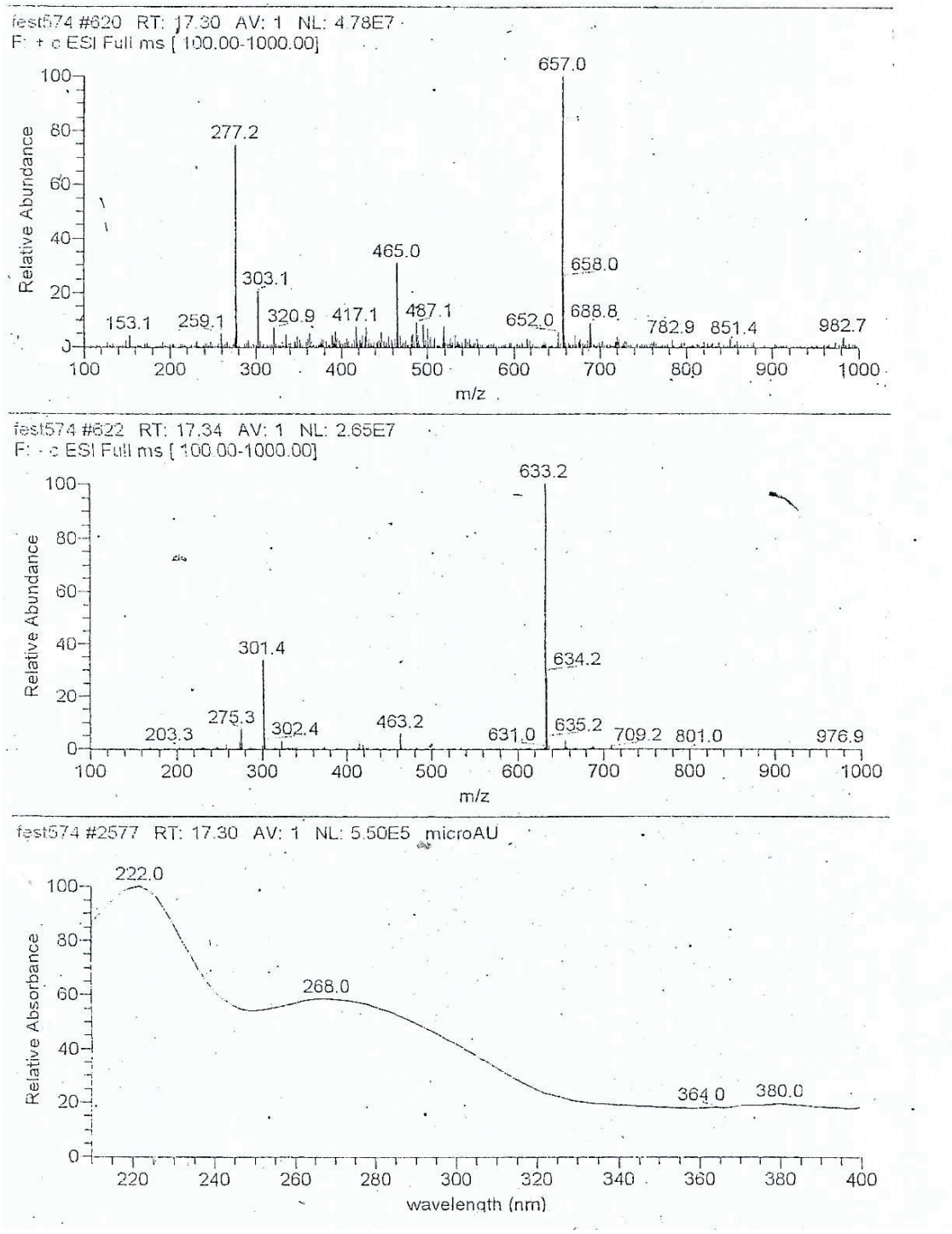

Figure 2b: HPLC-MS Spectrum (Positive mode) of Compound 1; HPLC-MS Spectrum (Negative mode) of Compound 1; UV Spectrum of Compound 1.

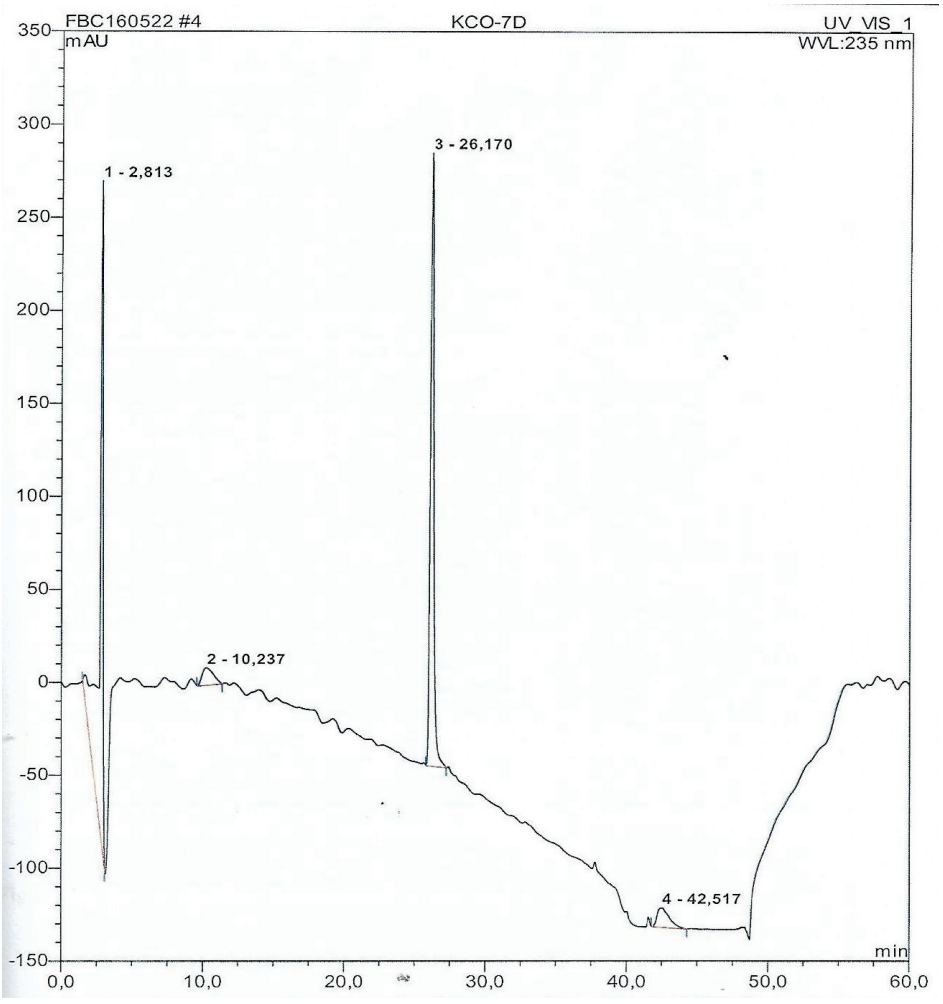

Figure 3a: HPLC Chromatogram of Compound 2

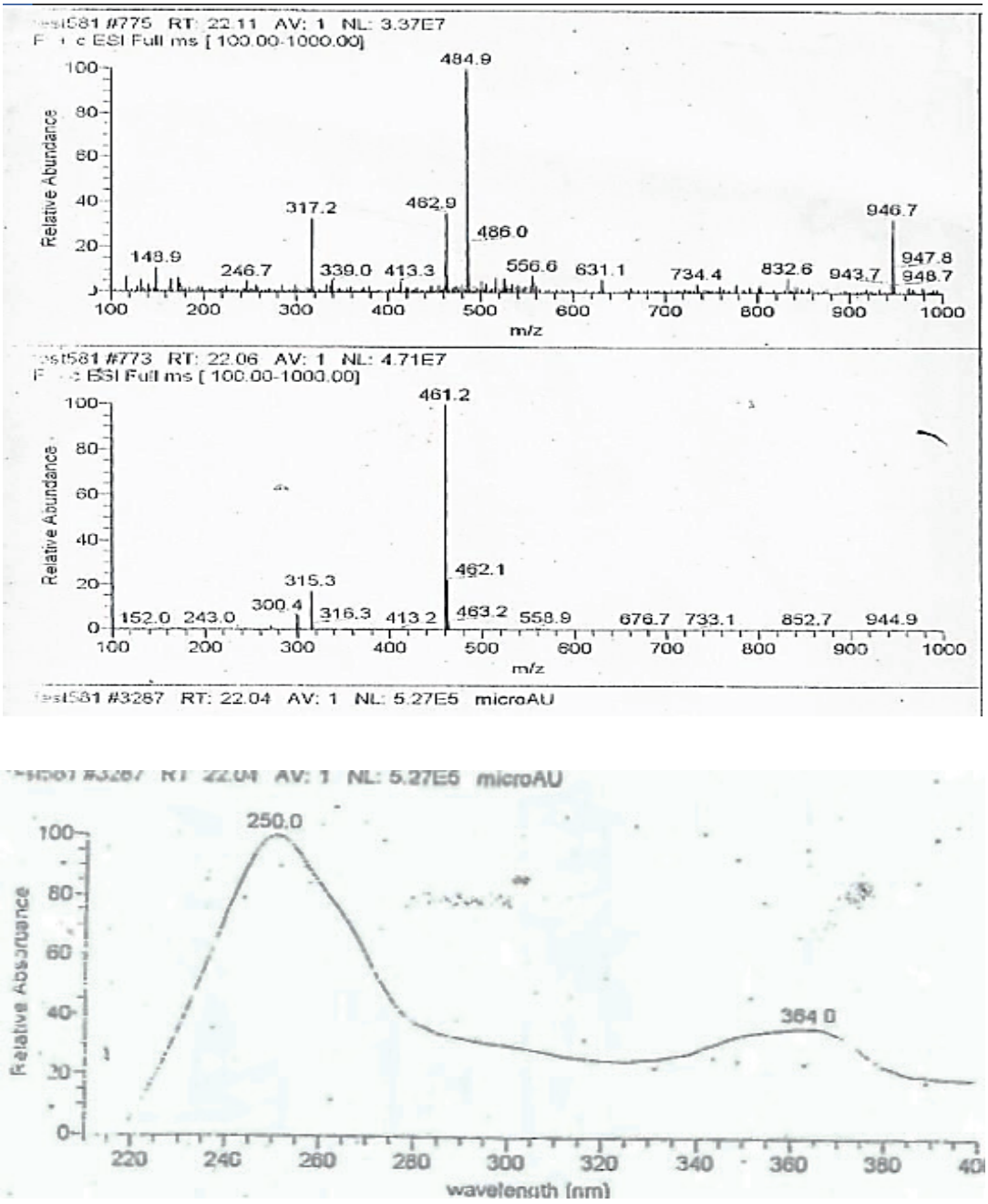

Figure 3b: HPLC-MS Spectrum (Positive mode) of Compound 2; HPLC-MS Spectrum (Negative mode) of Compound 2; UV Spectrum of Compound 2.

Effect of isolated compounds on in vitro Phagocytic ability of Neutrophils

There was a dose-dependent increase in the number of *Candida* cells phagocytosed by neutrophils compared to the control. At the dose of 100 µg/mL, compounds **1** and **2** elicited significant increase ($p < 0.01$) in phagocytic index (Figures 4a & 4b).

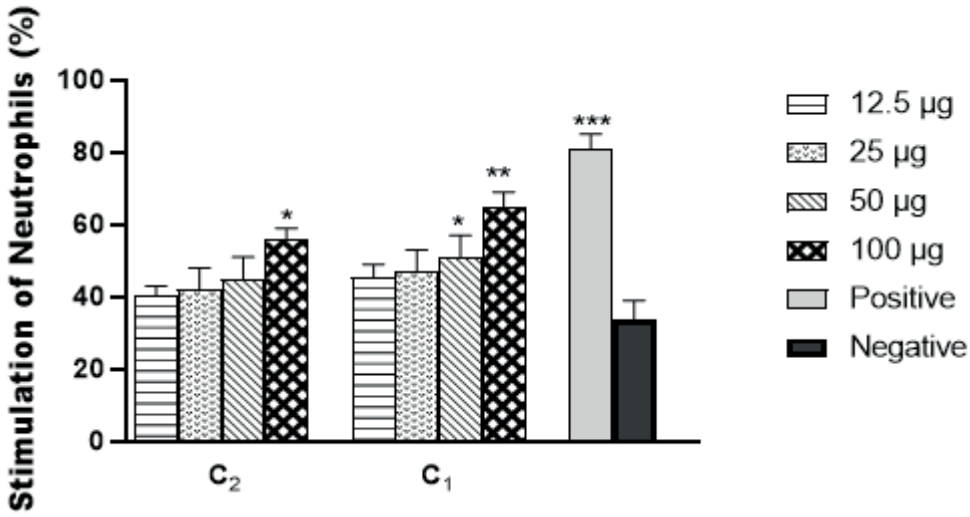

Figure 4a: Effects of the isolated compounds on *in vitro* Phagocytic ability of neutrophils, n= 5, **P<0.05*, *** P< 0.01, *** P< 0.001*. C_2 = compound 2, C_1 = compound 1, LPS= Lipopolysaccharide, PBS= Phosphate buffered solution

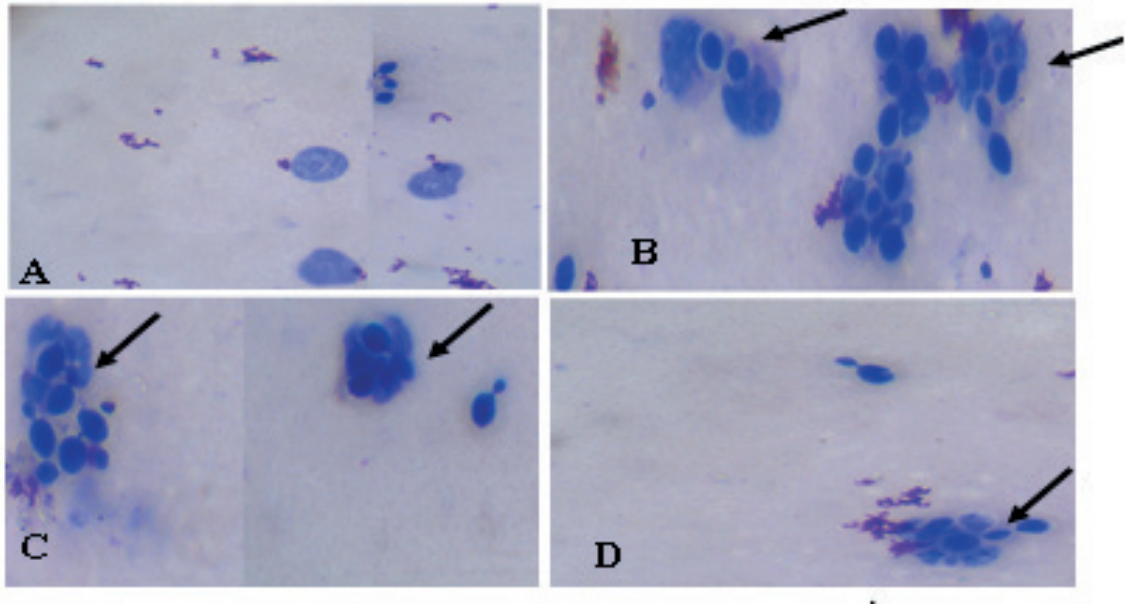

Figure 4b: Photomicrograph Phagocytosis of the isolated compounds. A = negative control. B = positive control (LPS), C = compound 1 and D = compound 2. Arrows reveal the neutrophils (multi-lobed) extending pseudopods to engulf *Candida* cells (spherical shape).

Effects of isolated compounds on Intracellular Killing Capacity of Neutrophils

All the treatment groups elicited a dose-dependent and significant increase in the number of NBT-positive cells relative to the control (Figures 5a & 5b).

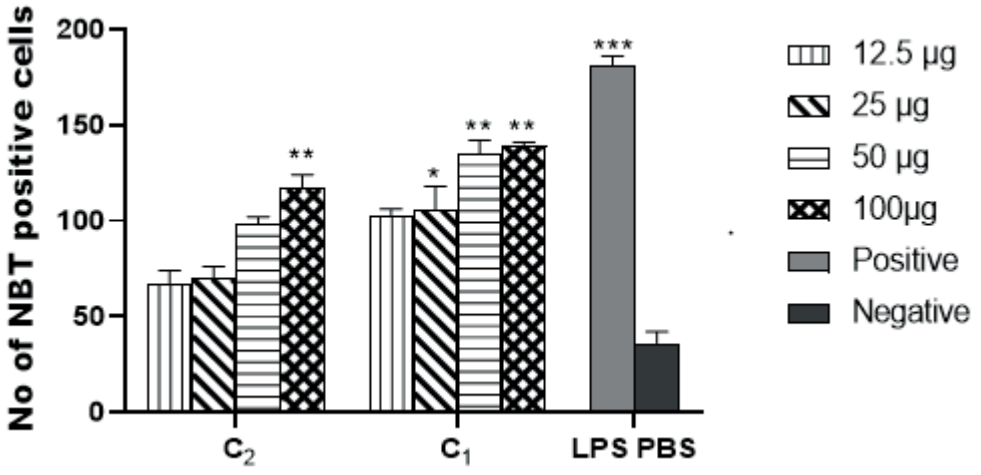

Figure 5a: Effects of the compounds on Intracellular killing capacity of neutrophils, n= 5, **P<0.05*, *** P< 0.01, *** P< 0.001*. C_2 = compound 2, C_1 = compound 1, LPS= Lipopolysaccharide, PBS= Phosphate buffered solution

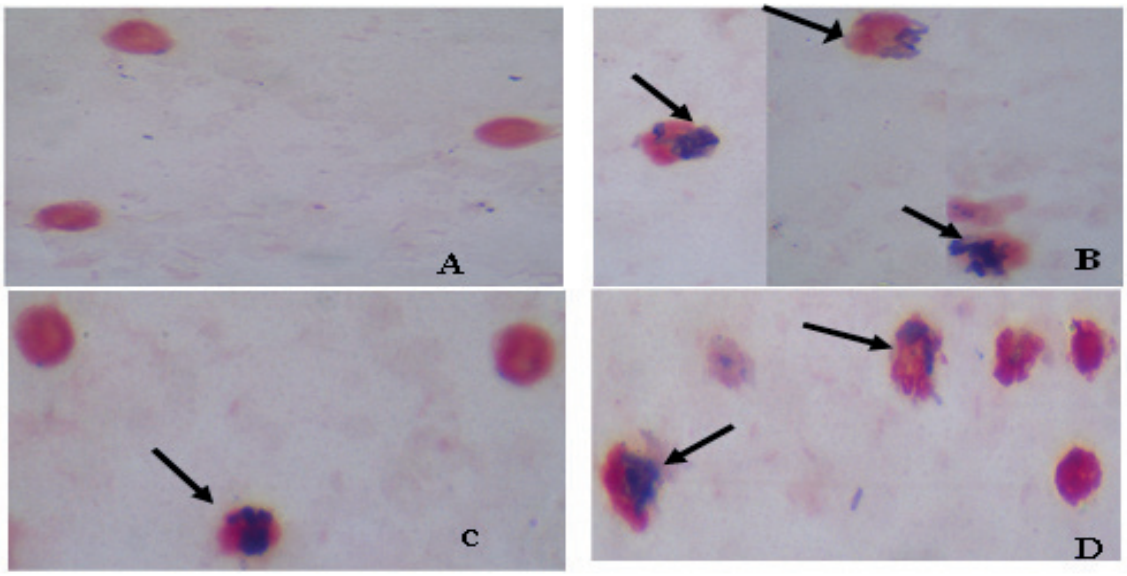

Figure 5b: Photomicrograph of NBT Positive Cells. A = negative control, B = positive control (LPS), C = compound 2 and D = compound 1. Arrows show the neutrophils (red) containing the red

Compound **1** was obtained as pale brown powder exhibiting a molecular ion peak at *m/z* 657.0 $[M+Na]^+$ in the positive mode and *m/z* 633 $[M-H]^-$ in the negative mode corresponding to a molecular formula of $C_{27}H_{22}O_{18}$. UV spectrum showed a characteristic band at λ_{max} of 222.0 and 268.0 nm suggesting the presence of a benzoic ring and C=O chromophore (Figure 1). HPLC-MS showed loss of gallic acid *m/z*

465 $[M-170]^+$ and subsequent loss of a hexose sugar *m/z* 303 $[M-170-162]^+$ (Figure 2). Galloyl moiety was further confirmed by the proton signal at δ_H 7.05 (s, 2H) assigned to H-2'/6' of gallic acid. Presence of the hexose unit was confirmed by the signals at δ_H 6.36 (d, J=2.16, 1H) assignable to the anomeric proton (H-1) (Table 1). ^{1}H NMR of the sugar moiety agrees with reported NMR data suggesting that the sugar was glucose, and the coupling constant of the anomeric proton supported the equatorial orientation of the anomeric proton, which is consistent with an α-D glucopyranoside configuration (Okoye et al. 2015; Jantan et al. 2014). Signals at δ_H 6.66 s (1H) and 6.69 s (1H) assignable to H-5" and H-5"" respectively suggested the presence of luteoyl moiety. Tannin glycoside (**1**) was thus deduced as 1-*O*-galloyl-6-*O*-luteoyl-α-D-glucopyranoside (Figure 1), which was previously reported (Subeki et al. 2005; Okoye et al. 2015).

Compound **2** was obtained as a pale brown powder. Structure of tannin glycoside (**2**) was deduced based on the HPLC-MS and UV spectra. UV spectrum showed two maxima at 250.0 and 364.0 nm, which is consistent with ellagic acid derivatives (Figure 3). HPLC-MS spectrum showed prominent peaks at *m/z* 484.9 $[M+Na]^+$ in the positive mode and 461.2 $[M-H]^-$ in the negative mode, which is consistent with a molar mass of 462 g/mol. Fragment ion at *m/z* 317.2 $[M-145]^+$ in the positive mode corresponds with a loss of deoxyhexose (rhamnose) with a corresponding 315.3 $[M-1-146]^-$ in the negative mode suggests the loss of aglycone moiety. α-rhamnose is the most abundant naturally occurring deoxyhexose, and we propose the presence of rhamnose and methyl ellagic acid derivatives. Tannin glycoside (**2**) is thus presented as 3-*O*-methylellagic acid 3'-*O*-α-rhamnopyranoside (Figure 1), which has been previously reported (Kim et al. 2001).

Phagocytosis and intracellular killing of invading microorganisms by macrophages and polymorphonuclear neutrophils constitute the body's primary line of defense. Phagocytes are powerful defense tools against invading microbes that threaten the life or functional integrity of the host (Splettstoesser and Schuff-Werner, 2002).

Phagocytes like neutrophils normally circulate with blood to migrate to the infection site (extravasation) and engulf any antigen. They first adhere firmly to the vascular endothelium so that they are not swept away by the sheer force of the flowing blood. Neutrophils bind firmly to the endothelium because the cell adhesion molecule on the neutrophil cell membrane called integrin is usually activated by chemoattractants secreted during infection to increase its affinity for the adhesion molecules on the vascular endothelium (Haddad, 2009). Bound neutrophils are afterward guided to the site of infection to cause phagocytosis by chemokines secreted by local macrophages at the injection site. Accumulation of neutrophils at the infection site leads to phagocytosis and, in addition to other mechanisms, leads to inflammation.

In this study, the adhesion of neutrophils to nylon fibers demonstrates a neutrophil attachment to the vascular endothelium and the number of neutrophils reaching the site of infection. Next step in phagocytosis after adhesion and migration of phagocytes to the infection site is the ingestion of the ingesting invader. Reticuloendothelial system (RES) is usually assessed by the rate at which carbon particles injected intravenously are cleared from the system. Overall phagocytic competence of neutrophils can be evaluated using the *in vitro* slide method, giving a reliable, quick, and easy assessment (Ganachari et al. 2004). *In vitro* evaluation of tannin glycoside **1** and **2** revealed a direct relationship between the concentration of the tannin glycoside and the phagocytic ability of neutrophils (Figures 4a & 5a). This is evident by the significant increase ($p < 0.01$) in the number of *C. albicans* cells engulfed by the treated neutrophils on the increasing dose. A comparison of the groups across various treatments showed a stepwise increase in phagocytic index, with **1** eliciting the highest phagocytic index, therefore revealing that its activity increases with pu-

rity. This suggests that some constituents might have antagonistic effects on the phagocytic pathway.

Final step in phagocytosis is ability of phagocytes to kill the engulfed pathogens intracellularly. It is one thing for phagocytosis to occur and another for the phagocyte to kill phagocytosed pathogens inside its cell. Competent phagocyte should be able to kill any engulfed pathogen. In some disease conditions like chronic granulomatous, phagocytes cannot kill some pathogens after engulfment because they cannot produce oxygen-dependent intracellular metabolites. These organisms remain inside the phagocytes and continue to grow and multiply, wreaking havoc on the body. Phagocytes kill pathogens using either toxic oxygen metabolites or lytic enzymes contained in their lysosomes. Present study showed that tannin glycoside **1** and **2** elicited a concentration-dependent and increase in the ability of neutrophils to kill within their cell the engulfed pathogens.

This experiment is based on the fact that during phagocytosis, a metabolic process known as respiratory burst activates a membrane-bound NADPH oxidase which catalyzes the generation of reactive oxygen radicals (O_2^-, OH, $^1O_2$, H_2O_2) that are microbicidal. We assayed for the generation of these toxic oxygen radicals by exposing our treated phagocytes (neutrophils) to nitroblue tetrazolium (NBT) dye – an oxidizing agent. This yellow dye gives a blue precipitate of formazan particles after a redox reaction with oxygen radicals. It was the presence of these blue deposits of reduced formazan in phagocyte solution that confirmed that our treated phagocytes became activated and generated oxygen radicals that reduced the NBT dye. Ability to produce and release within the cell large amounts of toxic oxygen radicals is an exceptional quality of activated mononuclear and polymorphonuclear phagocytes (Splettstoesser and Schuff-Werner, 2002). Therefore, compounds **1** and **2** not only increased phagocytosis as seen in the preceding model but also significantly stimulated the capacity to kill pathogens intracellularly in the treated neutrophils.

The varying degree of reduction of NBT by **1** and **2** confirms neutrophil's intracellular killing property and thus indicates immune-boosting effects. Intracellular infections cannot be killed by humoral immunity but by cell-mediated immunity, affected mainly by activated phagocytic and T cells. Cellular immunity also contributes significantly to the elimination of extracellular pathogens. Tannin glycosides isolated from *Phyllanthus* species have previously been reported to modulate various aspects of the immune system (Singh et al, 2017; Jantan et al, 2014; Jantan et al, 2019).

CONCLUSION

It is evident from these results that the tannin glycosides isolated from the leaves of *Phyllanthus muellerianus* not only have significant positive effects on phagocytosis via activation of neutrophils but also enhance their capacity to kill phagocytized pathogens. These compounds may be helpful '*leads*' in the development of agents for immune-deficiency diseases, antimicrobial agents, and vaccine adjuvants where they can enhance phagocytosis following opsonization by antibodies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

MNO: Conceptualization, Methodology, Investigation, Original draft preparation. **MOA**: Extraction of plant material, Investigation. **ONI**: Visualization, Investigation, Original draft preparation. **IP***:* Original draft preparation, Formal analysis. **FBCO**: Investigation, Formal analysis Validation. **PAA:** Supervision

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