Chemical constituents and bioactivities of *Glinus oppositifolius*

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ABSTRACT

Objectives: To isolate the secondary metabolites from the dichloromethane (DCM) extracts of *Glinus oppositifolius*; to test for the cytotoxicity of a new triterpene, oppositifolone (1); and to test for the hypoglycemic, analgesic, and antimicrobial potentials of 1, DCM and aqueous leaf extracts of *G. oppositifolius.* **Methods:** The compounds were isolated by silica gel chromatography and identified by nuclear magnetic resonance spectroscopy. The cytotoxicity potential of 1 was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Triterpene 1, DCM, and aqueous leaf extracts were tested for hypoglycemic potential using the oral glucose tolerance test; analgesic potential using the tail-flick assay, and antimicrobial potential using the disc diffusion method. **Results:** The DCM extracts of *G. oppositifolius* afforded 1, squalene, spinasterol, oleanolic acid, phytol, and lutein from the leaves; squalene and spergulagenin A from the stems; and spinasterol from the roots. Triterpene 1 was cytotoxic against human colon carcinoma 116 with an IC_{50} value of 28.7 but did not exhibit cytotoxicity against A549. The aqueous leaf extract at 200 mg/kg body weight (BW) exhibited hypoglycemic activity with a pronounced % blood glucose reduction of 70.76% ±17.4% within 0.5 h after introduction. The DCM leaf extract showed a lower % blood glucose reduction of 18.52% ±13.5% at 200 mg/kg BW within 1.5 h after introduction, while 1 did not exhibit hypoglycemic activity. The samples did not exhibit analgesic property and were inactive against multiple drug resistant bacterial pathogens. **Conclusion:** The compounds responsible for the hypoglycemic activity of *G. oppositifolius* which are fast acting (0.5 h) are found in the aqueous leaf extract.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, analgesic, antimicrobial, cytotoxic, *Glinus oppositifolius Linn,* hypoglycemic, *molluginaceae*, oppositifolone

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INTRODUCTION

Glinus oppositifolius Linn., locally known as papait is sold as a vegetable in the Philippines. It is reputed to exhibit anti-diabetes and anti-microbial properties. A number of studies has been conducted on the biological activities of crude extracts of *G. oppositifolius*. The 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay of the methanol extract of *G. oppositifolius* exhibited an IC_{50} > 1000 μg/ml (ascorbic acid, IC_{50} = 14.45 μg/ml), while in nitric oxide scavenging assay it gave an IC_{50} = 269 μg/ml (quercetin IC_{50} = 15.24 μg/ml).[1] A significant effect ($P < 0.05$) on oral glucose tolerance was noted at the doses of 200 mg/kg and 400 mg/kg body weight (BW) in mice. These results indicate that the methanol extracts of *G. oppositifolius* leaves possess moderate antioxidant activity and significant antihyperglycemic activity.[1] Another study reported that the methanol extract of *G. oppositifolius* was found to exhibit antioxidant activity which can be used for the treatment of oxidative stress related diseases.[2] Two pectin type polysaccharides, GOA1 and GOA2 from the aerial parts of *G. oppositifolius* were shown to exhibit

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potent dose-dependent complement fixating activities, and induced chemotaxis of macrophages, T cells and NK cells.[3] GOA1 was also reported to induce proliferation of B cells and the secretion of interleukin-1 β by macrophages, in addition to a marked increase of mRNA for interferon-γ in NK cells.[4] Two new triterpenoid saponins, glinosides A and B were isolated from the aerial parts of *G. oppositifolius*. Fractions of the extract exhibited better antiplasmodial activity than pure glinoside A.[5] Evaluation of α-glucosidase inhibitory activity of the aerial parts of *G. oppositifolius* led to the isolation of a new triterpene saponin, 16-O-(β-D-glucopyranosyl)-3 β,12 β,16 β,21α,22-pentahydroxyhopane and five known saponins, 3-O-(β-D-xylopyranosyl)-spergulagenin A, spergulacin, spergulin A, spergulacin A, and spergulin B. The new saponin exhibited the greatest inhibition of the enzyme with IC_{50} of 127 ± 30 μM. Kinetics study of this compound demonstrated mixed type of inhibition (Ki = 157.9 μM).[6] Moreover, treatment with ethanolic extract of aerial part of *G. oppositifolius* (200 mg/kg and 400 mg/kg) has reversed back the altered levels of biochemical markers in paracetamol induced hepatitis in rats to the near normal levels in a dose-dependent manner.[7] Another study reported that the methanolic extracts of *G. oppositifolius* possess central and peripheral analgesic and anti-inflammatory activity.[8] Furthermore, the alcoholic extract exhibited significant antimicrobial properties and anthelmintic activity against adult Indian earthworms, *Pheretima posithuma*.[9] The methanolic extract was also reported to significantly elicit a potent anticancer activity intraperitoneally at a dose of 2 mg/kg BW.[10] The whole plant of *G. oppositifolius* (L.) Aug. DC afforded L-(-)-(*N-trans*-cinnamoyl)-arginine, kaempferol 3-*O*-galactopyranoside, isorhamnetin 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-galactopyranoside, vitexin, vicenin-2, adenosine, and L-phenylalanine.[11]

In an earlier study, we reported the isolation of a new triterpene, oppositifolone (1) together with the known compounds, squalene (2), spinasterol (3), and lutein (6) from the dichloromethane (DCM) extract of the leaves of *G. oppositifolius*.[12] This study was conducted to investigate the chemical constituents of the DCM extracts of the stems, leaves, and roots of a local collection of *G. oppositifolius*. Furthermore, the cytotoxicity of 1 and the hypoglycemic, analgesic and antimicrobial potentials of 1, DCM and aqueous leaf extracts of *G. oppositifolius* were also tested.

We report herein the isolation of oleanolic acid (4) and phytol (5) in addition to our previously isolated compounds from the leaves of *G. oppositifolius*.[12] Moreover, the stems afforded 2 and spergulagenin A (**7**), while the roots yielded 3. The chemical structures (1–7) of the compounds isolated from the DCM extracts of *G. oppositifolius* are presented in Figure 1. We also report for the first time the cytotoxicity of 1 against human colon carcinoma (HCT 116) with an IC_{50} value of 28.7 μg/ml. We likewise report the hypoglycemic potential of the crude aqueous leaf extract at 200 mg/kg BW which showed a pronounced % blood glucose reduction of 70.76% ±17.4% at 0.5 h.

MATERIALS AND METHODS

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian NMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was conducted with silica gel 60 (70–230 mesh). Thin layer chromatography was performed with plastic-backed plates coated with silica gel F_{254}, and the plates were visualized by spraying with vanillin/H_2SO_4 solution, followed by warming.

Sample collection

The sample was collected from Villasis, Pangasinan, Philippines in January 2012. It was identified as *G. oppositifolius*

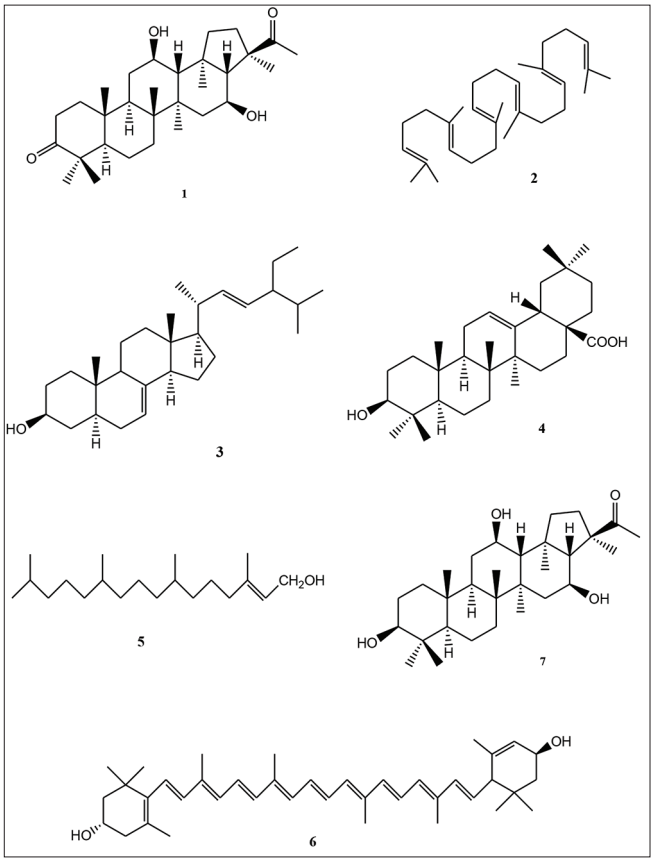

Figure 1: Chemical constituents of *Glinus oppositifolius*: Oppositifolone (1), squalene (2), spinasterol (3), oleanolic acid (4), phytol (5) and lutein (6) from the leaves; 2 and spergulagenin A (7) from the stems; and 3 from the roots

Linn. at the Bureau of Plant Industry, Manila, Philippines, A voucher specimen (#255) was deposited at the Chemistry Department, De La Salle University (DLSU), Manila, Philippines.

Preparation of aqueous leaf extract

The ground air-dried leaves of *G. oppositifolius* (50 g) were soaked in distilled water for 3 days and then filtered. The filtrate was freeze-dried to afford the crude aqueous extract (4 g).

Isolation of chemical constituents

The air-dried leaves (3 kg), stems (515 g), and roots (100 g) of *G. oppositifolius* were separately ground in a blender, soaked in DCM for 3 days and then filtered. The filtrates were concentrated under vacuum to afford the crude DCM extracts: Leaves (96.9 g), stems (6.2 g), and roots (3 g). The crude extracts were fractionated by silica gel chromatography using increasing proportions of acetone in DCM (10% increment) as eluents. A glass column 18 inches in height and 1.0 inch internal diameter was used for the fractionation of the crude extracts. Five milliliter fractions were collected. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until thin layer chromatography (TLC) pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Two milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

The 10–20% acetone in DCM fractions from the chromatography of the crude leaves extract were combined and rechromatographed (×2) in petroleum ether to afford 2 (18 mg). The 30% acetone in DCM fraction from the chromatography of the crude leaves extract was rechromatographed in 10% EtOAc in petroleum ether. The less polar fractions were rechromatographed (×2) in 5% EtOAc in petroleum ether to afford 5 (12 mg). The more polar fractions were rechromatographed (×4) in 5% EtOAc in petroleum ether to afford 3 (12 mg) after washing with petroleum ether. The 50% acetone in DCM fraction from the chromatography of the crude leaves extract was rechromatographed in CH_3CN: Et_2O: CH_2Cl_2 (0.5:0.5:9, v/v). The less polar fractions were rechromatographed (×3) in 15% EtOAc in petroleum ether to afford 4 (15 mg) after washing with petroleum ether. The more polar fractions were rechromatographed in CH_3CN: Et_2O: CH_2Cl_2 (0.5:0.5:9, v/v), followed by rechromatography (×2) in CH_3CN: Et_2O: CH_2Cl_2 (1:1:8, v/v) to afford 1 (25 mg) after washing with petroleum ether, followed by Et_2O. The 60% acetone in DCM fraction from the chromatography of the crude leaves extract was rechromatographed (×2) in CH_3CN: Et_2O: CH_2Cl_2 (0.5:0.5:9, v/v) to afford 6 (13 mg) after washing with Et_2O.

The DCM and 10% acetone in DCM fractions from the chromatography of the crude stems extract were combined and rechromatographed (×3) in petroleum ether to afford 2 (5 mg). The 60% acetone in DCM fraction from the chromatography of the crude stems extract was rechromatographed (×2) in CH_3CN: Et_2O: CH_2Cl_2 (0.5:0.5:9, v/v), followed by rechromatography in CH_3CN: Et_2O: CH_2Cl_2 (1:1:8, v/v) to afford **7 (**8 mg) after washing with petroleum ether, followed by Et_2O.

The 20–40% acetone in DCM fractions from the chromatography of the crude roots extract were combined and rechromatographed in 20% EtOAc in petroleum ether, followed by rechromatography (×2) in 12% EtOAc in petroleum ether to afford 3 (2 mg).

Cytotoxicity tests

Oppositifolone (1) was tested for cytotoxic activity against a HCT 116 cell line, a human lung nonsmall cell adenocarcinoma (A549) cell line and the noncancer cell line Chinese hamster ovary cells (AA8) at the Institute of Biology, University of the Philippines Diliman, Quezon City. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Doxorubicin was used as a positive control while dimethyl sulfoxide (DMSO) was used as a negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed.[13-16] The cells were plated into

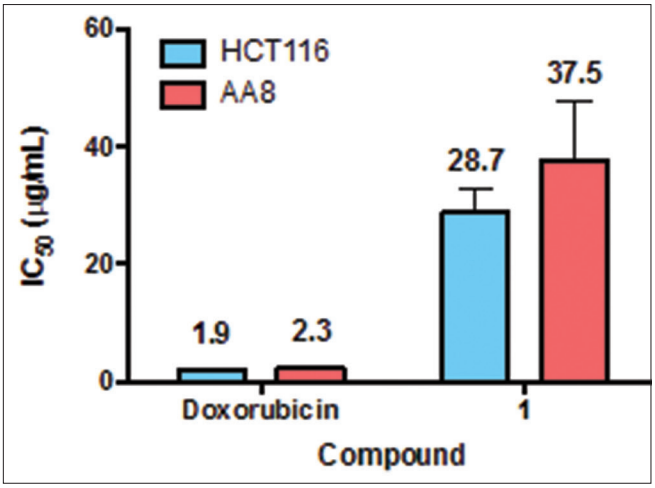

Figure 2: IC_{50} value of 1 and Doxorubicin against a human cancer cell line colon carcinoma 116 and a noncancer cell line Chinese hamster ovary cells (AA8)

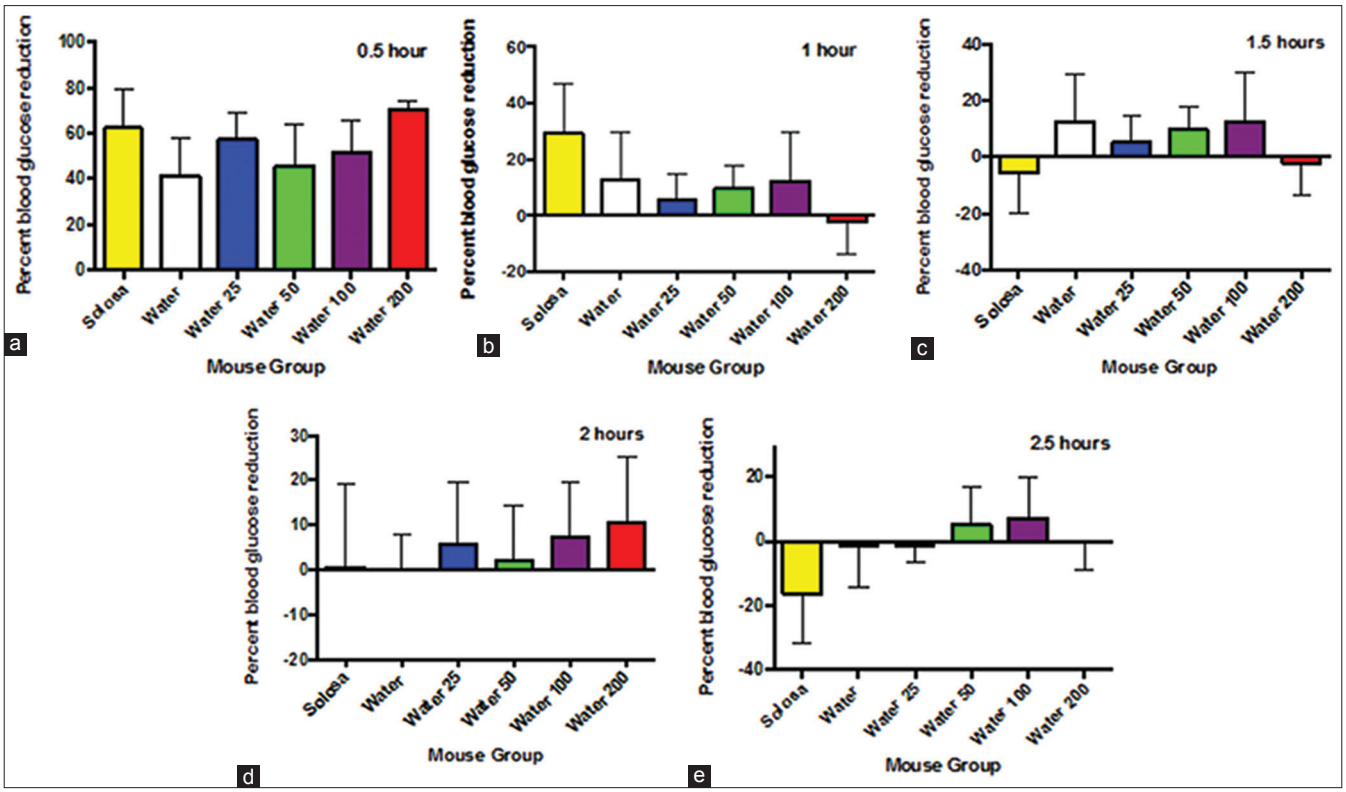

Figure 3: (a) Percent blood glucose reduction of aqueous extract at 25, 50, 100, and 200 mg/kg body weight taken at 0.5 h (b) Percent blood glucose reduction of aqueous extract at 25, 50, 100, and 200 mg/kg body weight taken at 1 h (c) Percent blood glucose reduction of aqueous extract at 25, 50, 100, and 200 mg/kg body weight taken at 1.5 h (d) Percent blood glucose reduction of aqueous extract at 25, 50, 100, and 200 mg/kg body weight taken at 2 h (e) Percent blood glucose reduction of aqueous extract at 25, 50, 100, and 200 mg/kg body weight taken at 2.5 h

a 96-well microtiter plate. The cell concentration was counted using a hemocytometer. Twenty milliliter of cell medium suspension with a concentration of 4×10^4 cells/ml was used for a microtiter plate. One hundred and ninety microliter of the suspension was transferred to each well. The microtiter plate was incubated in a humidifier incubator with 5% CO_2 for 24 h at 37°C. Four milligrams each of 1 and 2 were dissolved in 1 ml each of DMSO to make 4 mg/ml solutions. Compounds 1 and 2, doxorubicin, and DMSO were serially diluted. After 24 h of incubation, the cells were treated with 10 ml of 1 and 2 at 50, 25, 12.5 and 6.25 μg/ml. Three replicate wells for each concentration were used. The microtiter plate containing the treated cells was incubated for 72 h, after which the media from the wells were discarded and replaced with 20 ml of MTT dissolved in PBS (5 μg/ml) under low light intensity conditions. The plates were wrapped in aluminum foil and incubated at 37°C in 5% CO_2 for 24 h. After incubation, 100 ml of DMSO was added into each well to dissolve the MTT formazan crystals. Using an enzyme-linked immunosorbent assay reader, the absorbances were measured at wavelengths of 570 nm (measuring filter) and 620 nm (reference filter). Three trial assays were conducted with three replicate wells per concentration. The purple MTT formazan crystals produced from the experiment indicated the presence of live cells, since the yellow MTT-tetrazolium dye can be reduced by the mitochondria of living cells. Hence, a yellow product indicated cell death through either necrosis or apoptosis of the cancer cells. Absorbance readings were used to calculate the IC_{50} (concentration which resulted in a 50% reduction in cell viability) values of the samples using simple linear regression. The linear intrapolation/extrapolation method for sublethal toxicity: The inhibition concentration ICp approach (ICPIN Software Version 2, USEPA, Duluth, MN, USA) from a toxtat software program was used.

Hypoglycemic assay

Experimental animals

A total of 108 male albino mice (*Mus musculus* L.) of an inbred ICR strain (8 weeks old) weighing 23.0 ± 2.0 g was acclimatized for 7 days prior to conducting the bioassay. The animals were housed at the animal containment unit of DLSU-Manila with 12 h daylight and 12 h darkness, with free access to food pellets and water. A 16 h fasting period was conducted prior to each treatment procedure. Cervical dislocation was performed at the end of the animal treatment procedure. All procedures involving

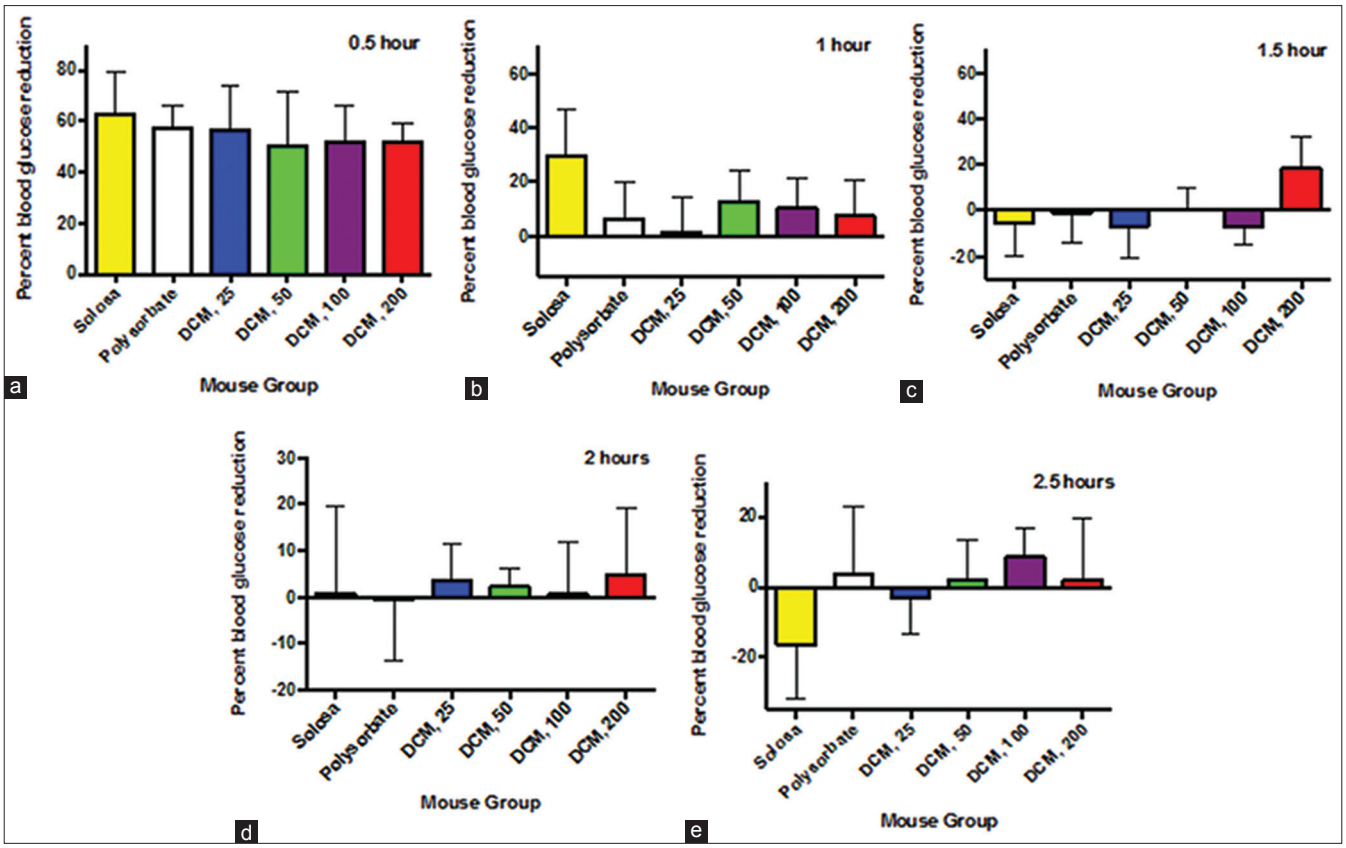

Figure 4: (a) Percent blood glucose reduction of dichloromethane extract at 25, 50, 100, and 200 mg/kg body weight taken at 0.5 h (b) Percent blood glucose reduction of dichloromethane extract at 25, 50, 100, and 200 mg/kg body weight taken at 1 h (c) Percent blood glucose reduction of dichloromethane extract at 25, 50, 100, and 200 mg/kg body weight taken at 1.5 h (d) Percent blood glucose reduction of dichloromethane extract at 25, 50, 100, and 200 mg/kg body weight taken at 2 h (e) Percent blood glucose reduction of dichloromethane extract at 25, 50, 100, and 200 mg/kg body weight taken at 2.5 h

animal handling were in accordance with the Philippine Association of Laboratory Animal Science code of practice for care and use of laboratory animals and with administrative order 40 of the Bureau of Animal Industry relative to Republic Act No. 8485.

Oral glucose tolerance test[17,18]

Oral glucose tolerance test (5 g/kg BW) was performed on normoglycemic mice ($n = 9$), followed by measurement of blood glucose level (mg/dl) using OneTouch Horizon (Lifescan, Johnson and Johnson, USA). Polysorbate 80 (25 ml/kg BW, UNIVAR, Australia) and distilled water were used as negative controls; Solosa (1.67 μg/kg BW, Glimepiride solosa, Aventis, Italy) dissolved in distilled H_2O was used as positive control; and crude aqueous leaf extract (200 mg/kg BW, 100 mg/kg BW, 50 mg/kg BW and 25 mg/kg BW) dissolved in distilled water, DCM leaf extract (200 mg/kg BW, 100 mg/kg BW, 50 mg/kg BW and 25 mg/kg BW) dissolved in Polysorbate 80 (25 mg/kg BW) and 1 (200 mg/kg BW, 100 mg/kg BW, 50 mg/kg BW, and 25 mg/kg BW) dissolved in polysorbate 80 (25 ml/kg BW) as test samples were orally administered to male albino mice. Blood glucose was measured within a 3 h period at 30 min intervals. Blood glucose reduction was computed and was used in the statistical analysis.

Statistical analysis

The results were analyzed using GraphPad Prism Version 6a (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance was performed to determine the significant effects of the hypoglycemic potentials of 1 and the aqueous and DCM extracts of *G. oppositifolis* leaves. The results were considered significant at $P < 0.05$. The difference between the pairs of group means was determined at 95% confidence interval using Tukey's multiple comparison test. Values are presented as mean ± standard deviation (SD).

Analgesic effect

The tail flick assay[19,20] was conducted on mice ($n = 7$) orally administered with Diclofenac (7.14 mg/kg BW) (GX International, Munti + nlupa City Philippines) and Polysorbate 80 (0.025 ml/g BW) as the positive and negative controls, respectively, and three dosages (50, 100 and 200 mg/kg BW) of aqueous leaf extract and DCM leaf extract and 1 dissolved in polysorbate 80.

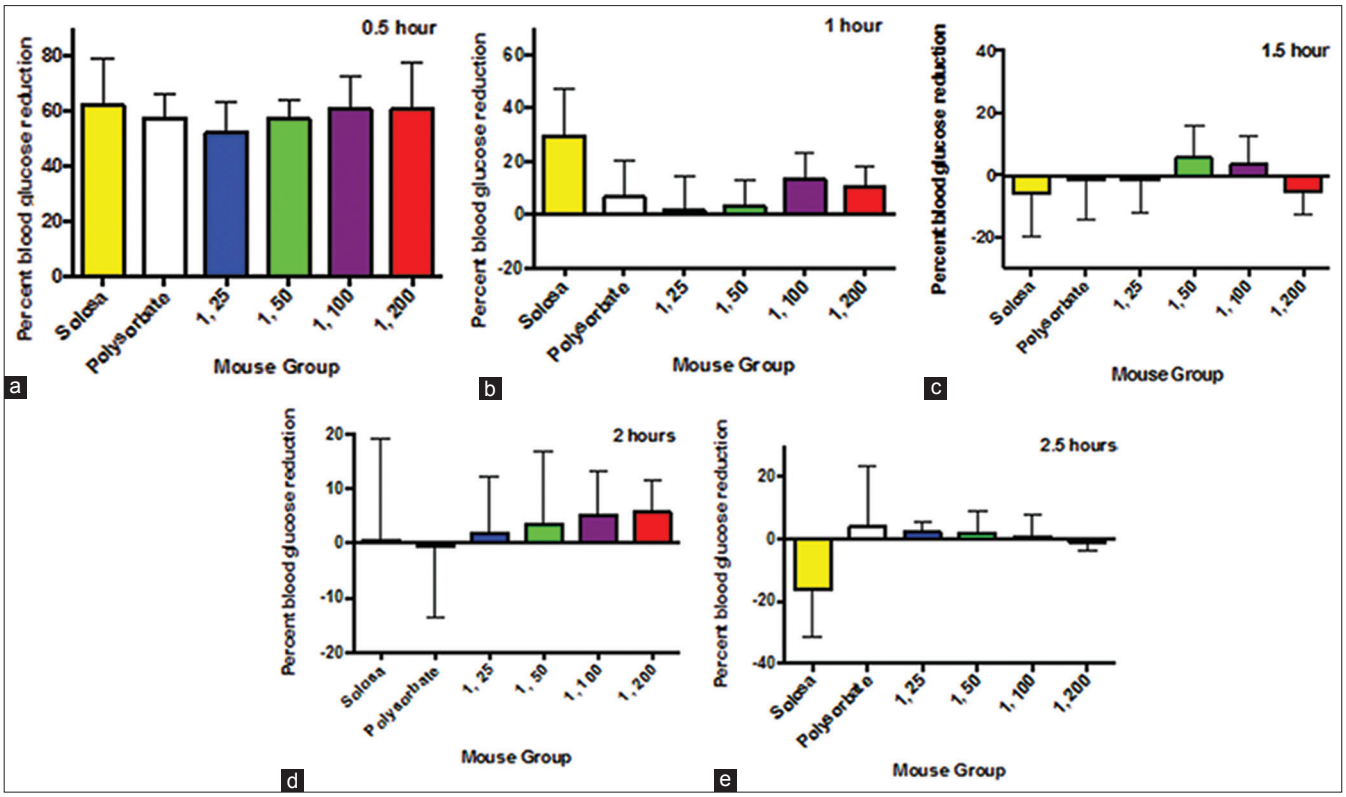

Figure 5: (a) Percent blood glucose reduction of 1 at 25, 50, 100, and 200 mg/kg body weight taken at 0.5 h (b) Percent blood glucose reduction of 1 at 25, 50, 100, and 200 mg/kg body weight taken at 1 h (c) Percent blood glucose reduction of 1 at 25, 50, 100, and 200 mg/kg body weight taken at 1.5 h (d) Percent blood glucose reduction of 1 at 25, 50, 100, and 200 mg/kg body weight taken at 2 h (e) Percent blood glucose reduction of 1 at 25, 50, 100, and 200 mg/kg body weight taken at 2.5 h

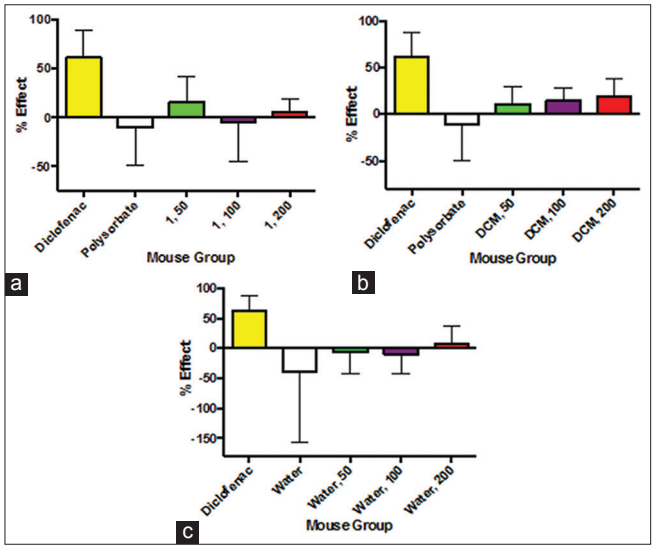

Figure 6: (a) Percent analgesic effect of 1 at 50, 100 and 200 mg/kg body weight (b) Percent analgesic effect of the dichloromethane extract at 50, 100, and 200 mg/kg body weight (c) Percent analgesic effect of the aqueous extract at 50, 100, and 200 mg/kg body weight

One-third of the distal part of the tail was immersed in a warm water bath (50°C) 1 h after the treatments. The time the mouse withdrew its tail from the water bath was noted. Percent inhibition was calculated according to the equation: % Analgesic effect = 100–([time that the experimental mice attempted to remove their tails/ average time that the control mice attempted to remove their tails] 100).

Antimicrobial test

Test bacterial isolates

The test bacterial isolates were taken from the DLSU Biology Department's microbial culture stocks that are maintained at −80°C. The purity of the cultures was ascertained, and the resistance phenotypes were confirmed using the standard disc diffusion method of Clinical and Laboratory Standards Institute (2010). The isolates comprised of the following: Extended spectrum β-lactamse-producing species of the family *Enterobacteriaceae* from nosocomial cases of the Philippine General Hospital Manila isolated in 2005;[21] Metallo-β-lactamase-producing *Pseudomonas aeruginosa* from nosocomial cases of the same hospital isolated in 2010;[22] Methicillin-resistant *Staphylococcus aureus* from prison inmates with furuncles and carbuncles isolated in 2007.[23] Forty-seven isolates made up of a combination of these strains belonging to seven genera, and eight species were included in the study. All the test isolates carry transferable genes coding for resistance

Table 1: Resistance phenotypes of test microorganisms from health care associated and community-acquired infections

Isolate ID	Resistance phenotype
Extended-spectrum β-lactamase producing *Enterobacteriaceae*[16]	
K. pneumonia 154	Atm, Caz, Ctx, Cro, Cfx, Fox, Te, Cip, Sxt, Na
K. pneumonia 226	Atm, Caz, Ctx, Cro, Cfx, Fox, Cfp, Ak, Tb
K. pneumonia 238	Atm, Caz, Ctx, Cro, Cfx, Tn, Sxt, Gm, Na, Ak
K. pneumonia 251	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Cip, Te
K. pneumonia 253	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Cip, Te
K. pneumonia 256	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Cip, Te
K. pneumonia 260	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Ak, Te
K. ozaenae 255	Atm, Caz, Ctx, Cro, Cfx, Ak, Tn, Cfp, Te
K. ozaenae 102	Atm, Caz, Ctx, Cro, Cfx, Fox, Ak, Tn, Cip, Te
K. ozaenae 224	Atm, Caz, Ctx, Cro, Cfx, Fox, Ak, Tn, Te
K. ozaenae 55	Atm, Caz, Ctx, Cro, Cfx, Fox, Ak, Tn, Te
K. ozaenae 10	Atm, Caz, Ctx, Cro, Cfx, Fox, Sxt, Tn, Te, Gm
K. ozaenae 64	Atm, Ctx, Cro, Cfx, Fox, Tn, Te, Gm, Ak, Cip, Na
K. ozaenae 166	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
E. cloacae 52	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Gm, Na
E. cloacae	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
E. cloacae 135	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Cip, Sxt, Gm, Na
E. cloacae 136	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Sxt, Gm, Na
E. coli 112	Atm, Caz, Ctx, Cro, Cfx, Tn, Te, Cip, Sxt, Gm, Ak, Na
E. coli 88	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
E. coli 92	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
P. mirabilis CTXM	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
E. aerogenes CTXM	Atm, Caz, Ctx, Cro, Cfx, Fox, Tn, Te, Ak, Cip, Sxt, Na
Methicillin resistant *S. aureus*[18]	
S. aureus MCJA12	Resistant to all β-lactam antibiotics
S. aureus MCJA24	Resistant to all β-lactam antibiotics
S. aureus MCJA26	Resistant to all β-lactam antibiotics
S. aureus MCJA29	Resistant to all β-lactam antibiotics
S. aureus NBP2-1	Resistant to all β-lactam antibiotics
S. aureus NBP-4	Resistant to all β-lactam antibiotics
S. aureus NBP7-1	Resistant to all β-lactam antibiotics
S. aureus NBP16	Resistant to all β-lactam antibiotics
S. aureus NBP17-1	Resistant to all β-lactam antibiotics
S. aureus NBP40	Resistant to all β-lactam antibiotics
Metallo-β lactamase-producing *P. aeruginosa*[17]	
P. aeruginosa 23	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz
P. aeruginosa 30	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Ak
P. aeruginosa 32	Tc, Imi, Mem, Gm, Tn, Ak, Cip, Lev
P. aeruginosa 84	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Ak
P. aeruginosa 123	Tc, Caz, Cpm, Imi, Mem, Ptz,
P. aeruginosa 150	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Atm
P. aeruginosa 192	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Atm, Ak
P. aeruginosa 200	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Ak
P. aeruginosa 205	Tc, Imi, Mem
P. aeruginosa 232	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Ak
P. aeruginosa 260	Tc, Caz, Cpm, Imi, Cip, Lev, Atm
P. aeruginosa 263	Tc, Caz, Cpm, Imi, Mem, Atm, Gm, Tn, Ak, Cip, Lev, Ptz
P. aeruginosa 264	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Atm, Ak
P. aeruginosa 266	Tc, Caz, Cpm, Imi, Mem, Gm, Tn, Cip, Lev, Ptz, Atm, Ak
Antibiotic susceptible reference strains	
E. coli ATCC 25922	
S. aureus ATCC 25923	

Ak=Amikacin; Atm=Aztreonam; Caz=Ceftazidime; Cfp=Cefoperazone; Cfx=Cefuroxime; Cip=Ciprofloxacin; Cpm=Cefepime; Cro=Ceftriaxone; Ctx=Cefotaxime; Fox=Cefoxitin; Gm=Gentamicin; Imi=Imipenem; Lev=Levofloxacin; Mem=Meropenem; Na=Nalidixic acid; Ptz=Piperacillin-tazobactam; Sxt=Sulfamethoxazole-trimethoprim; Tc=Ticarcillin; Te=Tetracycline; Tn=Tobramycin; *K. pneumonia=Klebsiella pneumonia; K. ozaenae=Klebsiella ozaenae; E. cloacae=Enterobacter cloacae; E. coli=Escherichia coli; P. mirabilis=Proteus mirabilis; E. aerogenes=Enterobacter aerogenes; P. aeruginosa=Pseudomonas aeruginosa*

to multiple antimicrobial agents [Table 1]. Screening for inhibitory activities was also done on the drug-susceptible reference strains *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923.

Screening for the presence of antimicrobial activities

After confirmation of their resistance phenotypes, the test strains were grown in brain heart infusion agar (BHIA) plate at 37°C for 16–18 h. Colonies were

suspended in sterile 0.9% NaCl, and turbidity of the culture was adjusted to equal that of 0.5 MacFarland turbidity standard. The inoculum was swabbed unto BHIA plates, after which sterile filter paper discs, each impregnated with 20 μL of 1 or DCM leaf extract or aqueous leaf extract and allowed to air dry, were introduced into the inoculated agar surface. The well-diffusion method was also used to assay for antimicrobial activities. Negative control discs and wells contained either distilled water, 95% ethanolic (EtOH) or DCM. Each assay was done in triplicate. The production of a zone of inhibition around the disk would denote the presence of antimicrobial activity of 1, DCM and aqueous leaf extracts.

Different preparations from the plant were tested. Triterpene 1 and the DCM leaf extract were found to be very hydrophobic, and did not totally dissolve in 95% EtOH and DCM. To allow diffusion of the test substances into the culture-inoculated BHIA, Tween 80 in concentrations of 0.5% and 1.25% were added into BHIA medium, and to 1 (in 95% EtOH or DCM) and the DCM leaf extract, respectively. Triterpene 1 (in 95% EtOH or in DCM), DCM and aqueous leaf extracts were tested at concentrations of 30, 150, 450, 1500, 3000, 4000, 5000, 6000, and 10,000 μg/ml. Five of the multiple drug resistant test isolates, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were sent to the Microbial Culture Collection at the Natural Sciences Research Institute of the University of the Philippines (UP-NSRI), Diliman, Quezon City for confirmation of the results.

RESULTS AND DISCUSSION

The DCM extracts of the air-dried *G. oppositifolius* afforded spergulagenin A (7) and squalene (2) from the stems; 2, oppositifolone (1), spinasterol (3), oleanolic acid (4), phytol (5) and lutein (6) from the leaves; and 3 from the roots. The structures of 1–7 were identified by comparison of their ^{1}H NMR and/or ^{13}C NMR data with those reported in the literature for oppositifolone (1),[12] squalene (2),[24] spinasterol (3),[25] oleanolic acid (4)[26,27] phytol (5),[28] lutein (6),[29] and spergulagenin A (7).[30]

Cytotoxic activity of 1

Oppositifolone 1 was evaluated for cytotoxicity against the human cancer cell lines, nonsmall cell lung adenocarcinoma (A549) and HCT 116, and the noncancer cell line Chinese hamster ovary cells (AA8) using the MTT cytotoxicity assay. Triterpene 1 was cytotoxic against HCT 116 with IC_{50} value of 28.7 μg/ml, while Doxorubicin exhibited an IC_{50} value of 1.9 μg/ml [Figure 2]. Triterpene 1 and Doxorubicin were also cytotoxic against AA8 with IC_{50} values of 37.5 μg/ml and 2.3 μg/ml, respectively. Triterpene 1 had no linear interpolation with the A549, thus, IC_{50} could not be calculated. This implied that 1 did not exhibit cytotoxic effect against this cell line.

Hypoglycemic activity

The hypoglycemic potential of 1, DCM and aqueous leaf extracts were observed in groups of mice administered with the test samples, positive control (Solosa), and negative controls (polysorbate 80 and water) within a 3 h blood glucose measurement period at 0.5 h intervals. The percent blood glucose reductions of aqueous leaf extracts at 25, 50, 100, and 200 mg/kg BW taken within a 3 h blood glucose measurement period at 0.5 h intervals are presented in Figure 3a-e. Solosa was observed to be a fast acting hypoglycemic agent with a relatively short duration of activity. Solosa was able to reduce blood glucose levels at an average rate of 62.27 ± 16.8% for the first 0.5 h of measurement [Figure 3a] and 29.46% ±17.4% for the 1st h of measurement [Figure 3b]. Such a percentage reduction is concomitant with the reported physiological effects of Solosa on blood glucose reduction.[31] At 0.5 h, the positive control (Solosa) is significantly different ($P < 0.05$) from the negative control (water) [Figure 3a]. The administration of aqueous leaf extract at a concentration of 200 mg/kg BW share almost the same pattern of blood glucose reduction as Solosa, with a pronounced % reduction of 70.76 ± 17.4% within 0.5 h after introduction of the aqueous leaf extract [Figure 3a]. The aqueous leaf extract at a concentration of 200 mg/kg BW is significantly different ($P = 0.0007$) from the negative control (water) and exhibited an immediate activity similar to the positive control [Figure 3a]. In the next 1 h and 1.5 h, the blood glucose reduction may be due to insulin since the % reduction for the aqueous leaf extract and the negative control (water) are similar [Figure 3b and c].

The percent blood glucose reductions of DCM leaf extracts at 25, 50, 100, and 200 mg/kg BW taken within a 3 h blood glucose measurement period at 0.5 h intervals are presented in Figure 4a-e. For testing the hypoglycemic potential of the DCM leaf extract, the extract was dissolved in Polysorbate 80 (negative control). At 0.5 h, all the samples tested gave similar % blood glucose reduction [Figure 4a]. At 1 h, the % blood glucose reduction of Solosa (29.46% ±17.4%) is significantly different ($P < 0.05$) from the negative control (6.49 ± 13.6%) [Figure 4b]. The administration of DCM leaf extract at a concentration of 200 mg/kg BW gave a % reduction of 18.52% ±13.5%, 1.5 h after introduction of the DCM leaf extract [Figure 4a] which is significantly different ($P < 0.05$) from polysorbate 80 (−1.35 ± 12.7%) [Figure 4c]. Thus, the DCM leaf extract

exhibited lower (18.52% ±13.5%) hypoglycemic activity than the aqueous leaf extract (70.76% ±17.4%) which was also faster acting (0.5 h).

The percent blood glucose reductions of 1 at 25, 50, 100 and 200 mg/kg BW taken within a 3 h blood glucose measurement period at 0.5 h intervals are presented in Figure 5a-e. Oppositifolone (1) at different concentrations (25, 50, 100, and 200 mg/kg BW) did not exhibit hypoglycemic activity since it did not show significant difference in % blood glucose reduction when compared to the negative control (Polysorbate 80).

Analgesic effect

The tail flick assay specifically tests for centrally mediated perception of pain by inhibiting certain opioid receptors.[32] The results obtained from this assay [Figures 6a-c] showed that the thermal response of the positive control, diclofenac has reduced the perception of pain in the experimental animals. The % analgesic effect of diclofenac (61.3 ± 27) is significantly different ($P < 0.05$) from Polysorbate 80 (−10.4 ± 39.0) [Figure 6a and b]. The % analgesic effect of diclofenac (61.3 ± 27) is also significantly different ($P < 0.05$) from water (38.7 ± 116.9) [Figure 6c]. However, A and the DCM and aqueous leaf extracts at 50, 100, and 200 mg/kg BW did not exhibit analgesic activity since they did not show % analgesic effect which is significantly different from the negative control, polysorbate 80 [Figure 6a-c].

Antimicrobial activity of 1, dichloromethane leaf extract and aqueous leaf extract

The resistance phenotypes of the 49 test bacterial isolates were confirmed using the disc diffusion method and are shown in Table 1. Results of the assays using triterpene (1) in 95% EtOH and in DCM, the DCM and aqueous leaf extracts in concentrations that ranged from 30 μg/ml to 10 mg/ml showed the absence of antimicrobial activities against all the test samples. The same results were obtained by UP-NSRI on the bacterial isolates sent to them for testing.

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