Pharmacogn. Mag.
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ORIGINAL ARTICLE

Immunomodulatory and Cytotoxic Properties of Natural Triterpenoids Isolated from *Grewia flavescens* Juss

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Submitted: 18-Jun-2020 Revised: 06-Jul-2020 Accepted: 10-Mar-2021 Published: 10-Jun-2021

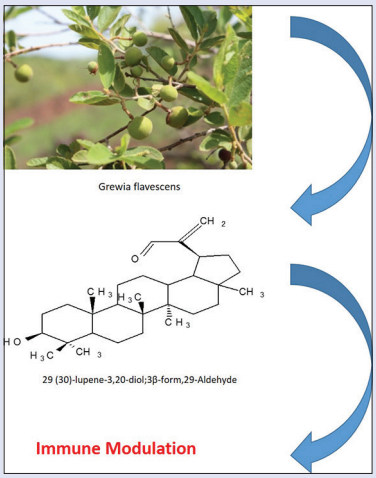
ABSTRACT

Background: *Grewia flavescens* Juss (*Malvaceae*) is a species that is distributed throughout semi-arid and sub-humid tropical areas of Africa, Saudi Arabia, Yemen, and India. It is used in various traditional herbal practices for the treatment of various disease conditions. **Objectives:** The present investigation was undertaken to examine the immunomodulatory and cytotoxic properties of natural triterpenoids isolated from the stem bark of *G. flavescens*. **Materials and Methods:** The immunomodulatory activity was measured using oxidative burst chemiluminescence and phytohemagglutinin stimulated T-cell proliferation assay. The cytotoxicity was measured using MTT assay. One new triterpenoid (29 [30]-lupene-3, 20-diol; 3 β-form, 29-Aldehyde [4]) in addition to four known ones; lupeol (1), ß-sitosterol (2), betulin (3), and ß-sitosterol glucopyranoside (5), have been isolated from the stem bark of *G. flavescens* and identified using Fourier-transform infrared spectroscopy, ultraviolet, and NMR techniques. **Results:** Chemiluminescence experiments showed that no compound exerted an inhibitory effect on reactive oxygen species production. However, 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) significantly repressed the cell proliferation activity with an IC_{50} of 8.7 μg/mL, whereas, ß-sitosterol glucopyranoside (5) and ß-sitosterol (2) showed moderate inhibitory activities (IC_{50} of 16.7 and 23.6 μg/mL, respectively) compared to the standard drug prednisolone (0.2 μg/mL). Betulin (3) revealed the highest cytotoxicity activity toward Hela cancer cells, followed by 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4), and ß-sitosterol (2) with IC_{50} 11.10, 16.5 and 21.51 μg/ml respectively. **Conclusion:** The present investigation supports the traditional use of this plant in treating various diseases and it has a good potential for future studies based on animal model based, which could bring more mechanistic facts about this plant's activity.

Key words: Cytotoxicity, *Grewia flavescens* Juss, immunomodulation, oxidative burst, T-cell proliferation, triterpenoids

SUMMARY

- *Grewia flavescens Juss* (*Malvaceae*) is a species that is used in various traditional herbal practices for the treatment of various disease conditions
- We have isolated one new triterpenoid in addition to four known triterpenoids from the *G. flavescens* Juss, which were evaluated for immunomodulatory and cytotoxicity properties
- The new compound; (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde significantly interfere with the adaptive immune response (T cell proliferation). It inhibited the phytohemagglutinin-activated T-cell.



Abbreviation used: AIDS: Acquired immune deficiency syndrome; ATCC: American type culture collection; $CHCl_3$: Chloroform; COSY: Correlation spectroscopy; FT IR: Fourier-transform infrared spectroscopy; HMBC: Heteronuclear multiple bond coherence; HMQC: Heteronuclear multiple quantum coherence; MeOH: Methanol; P. E: Petroleum ether; PBMC: Peripheral blood mononuclear cells; PHA: Phytohemagglutinin; PMS: Polymorph nuclear cells; ROS: Reactive oxygen species; SOZ: Serum-opsonized zymosan; TLC: Thin-layer chromatography

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DOI: 10.4103/pm.pm_235_20

Access this article online
Website: www.phcog.com
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INTRODUCTION

The immune system plays a vital role in monitor tissue homeostasis by the maintenance of the host defense system against pathogens and in cancer prevention. Immunomodulating drugs are needed for treating many diseases/conditions such as infections, organ transplantation, cancer, rheumatoid arthritis, systemic lupus erythematosus, down syndrome, Crohn's autoimmune diseases, and the acquired immune deficiency

Cite this article as: ELhassan GO, Yagi S, Mesaik MA, Mohan S, Alhazmi HA, Al-Bratty M, *et al*. Immunomodulatory and cytotoxic properties of natural triterpenoids isolated from *Grewia flavescens* Juss. Phcog Mag 2021;17:S9-14.

syndrome.[1] The interest in medicinal plants in immunomodulation has been increasing due to its contents of structurally diverse phytochemicals, which were found to have been involved in the modulation of phagocytes such as neutrophils, monocytes, macrophages, and phagocytes.[2] Large volume of reactive oxygen species (ROS) is released by these phagocytes by pathogens stimulation through a process called a respiratory oxidative burst.[3] This mechanism positively impacts the immune response.[4] The release of excessive volumes of ROS has severe implications in the pathogenesis of several inflammatory disorders and related diseases.[4,5] Hence, plant-derived compounds with immune-modulatory properties for the use of clinically useful drug development become a major goal of herbal research laboratories.

Grewia flavescens Juss belongs to the family *Malvaceae*. The species is distributed throughout semi-arid and sub-humid tropical areas of Africa, Saudi Arabia, Yemen, and India.[6] In Sudan, the root bark of this plant is been used in traditional medicine to treat diarrhea. It is also considered as an aphrodisiac.[7] In Indian folk medicine, *G. flavescens* is used as anthelmintic, CNS depressant,[8] anti-inflammatory, antimalarial, antidiabetic, and analgesic.[9] In South Africa, the plant is used traditionally as an antidiabetic medicine.[10] In some parts of Sudan, the fruit is used as a general tonic especially during the fasting month of Ramadan.[11] The previous phytochemical investigations of *G. flavescens* have resulted in the isolation of some triterpenoids, i.e., α-amyrin, β-amyrin, betulin, friedelin, triacontanol, and β-sitosterol.[8] Triterpenes are naturally occurring compounds with growing interest as phytosterols due to their wide spectrum of biological activities.[12,13] Triterpenes, which contain a broad chemical group of active principles, are implicated in the pharmacological mechanism of action of many herbs used in folk medicine. In addition, to be known as immunomodulation agent, it also is known for other significant pharmacological activities such as antimicrobial, antiviral, and anticancer.[14]

The presence of triterpenoids and compounds which were found in *G. flavescens* has been also found in different plants from *Grewia* species. For instance, *Grewia asiatica* which is a traditional medicinal plant, with high presence of triterpenoids, is known to exhibit anticancer and immune-modulatory activities.[15,16] In efforts to screen anticancer plants from the African region in our laboratory, we have observed significant cytotoxicity exerted by *G. flavescens* extract. Looking into these facts and the *G. flavescens*'s traditional use we propose that the triterpenoids of *G. flavescens* could also exhibit similar activity, especially in modulating the immune system. Hence, in the present study, we have isolated triterpenoids from *G. flavescens and* investigated their immune-modulatory and cytotoxicity activities.

MATERIALS AND METHODS

Plant material

The stem bark of *G. flavescens* was collected in the month of January from Kordofan region of Western Sudan. The plant was botanically identified and a voucher specimen number (GF/709) was provided, then deposited in the Herbarium of Botany Department, University of Khartoum.

Extraction and isolation

The dried and ground stem bark of *G. flavescens* (500 g) was extracted by maceration in petroleum ether and ethanol for 5 days with occasional shaking. Both extracts were filtered and concentrated under reduced pressure using a rotary evaporator. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (DC-Alufolien 60 F_{254} of E. Merck) and spots were located by ceric sulfate spraying reagent.

The ethanol extract (11 g) was subjected to flash column chromatography (CC) on silica gel (silica gel 60, 70–230 mesh; Merck; 600 g) using a stepwise gradient elution of n-hexane to EtOAc and to methanol [MeOH]. A total number of 80 fractions (50 mL each) were collected and finally, 8 fractions were obtained on combining the eluates according to their similarity behavior on TLC. Fractions 2 (113 mg), 3 (120 mg), and 5 (122 mg) were applied repeatedly to silica gel CC and eluted with petroleum ether (P. E): EtOAc gradients to afford pure lupeol (1) (15 mg) and ß-sitosterol (2) (30 mg) in P. E: EtOAc (8:2), Fractions 4 (56 mg) and 5 (175 mg) were subjected to repeated flash chromatography using chloroform ($CHCl_3$): EtOAce mixture of increasing polarity as eluent to obtain Betulin (3) (15 mg) and 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) (20 mg). Fraction 8 (601 mg) was subjected to repeated flash chromatography using $CHCl_3$: MeOH mixture to afford ß-sitosterol glucopyranoside (5) (15 mg). All isolated compounds were over 95% pure as estimated by ^{1}HNMR.

Structure elucidation

The FT-IR spectra were recorded on a vector 22 instrument. ultraviolet was recorded on Unicam Helos BVI 14 model No. 053925. The ^{1}H NMR and 2D NMR spectra (correlation spectroscopy [COSY], heteronuclear multiple quantum coherence [HMQC], and heteronuclear multiple bond coherence [HMBC]) were recorded on a Bruker AMX 500 MHz and 100 MHz NMR (Avance) instruments using the UNIX data solvent. EI-MS spectra were recorded on a Finnigan MAT 312. FAB mass measurements were performed on Jeol JMS HX 110 mass spectrometer using glycerol as the matrix. HREI MS was carried out on Jeol JMS 600 mass spectrometer.

Oxidative burst assay

Luminometer (RS Labsystems Luminoskan, Finland) was used in this assay. Two milliliters of pooled human serum was mixed with 30 mg of zymogens (Sigma Chemical Co, St. Louis, MO) to prepare the serum-opsonized zymosan (SOZ). The volume was then make up to 10 ml with the addition of Tris base NaCl. The obtained mixture was then vortexed and incubated at 37°C in a shaker water bath for 30 min, followed by centrifugation at 2000 g at room temperature for the final wash to obtain the pellet by removing the supernatant. The pellet was

Table 1: Chemiluminescence effect, PHA- activated T-cell proliferation and cytotoxicity activity for the isolated compounds.

Compound	ROS (IC_{50} µg/mL)*		T-cell proliferation (IC_{50} µg/mL)*	Cytotoxicity activity (IC_{50} µg/mL)*	
	Whole blood	Neutrophil		Normal cell	Cancer cell
Lupeol (1)	> 100	> 100	> 50	> 100	23.5±1.7
ß-sitosterol (2)	> 100	> 100	23.6±1.2	> 100	21.5±1.5
Betulin (3)	> 100	> 100	>50	> 100	11.1±0.5
29 (30)-lupene-3, 20-diol; 3β-form, 29-Aldehyde (4)	> 100	> 100	8.7±0.3	> 100	16.5±0.5
ß-sitosterolglucopyranoside (5)	> 100	> 100	16.7±1.3	> 100	>100
Ibuprofen	11.2±1.9	2.8	-	-	-
Prednisolone	-	-	0.2±0.3	-	-
Cyclohexamide	-	-	-	0.13±0.02	-

*IC_{50} values are expressed as mean of three determinations.

re-suspended in 10 ml of Tris base NaCl. 0.037% concentration of SOZ was achieved in each experimental wells.[17]

Luminol (Research Organics Cleveland, OH, USA) was added with borate buffer to prepare the luminol solution as described earlier.[18] To perform the assay, heparinized blood was collected from the healthy volunteers aged 25–45 years. The whole study was conducted as per the guidelines from the International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan (UoK, No: ICCBS/IEC-009-BC-2017/Protocol/1.0.). The volunteers were well informed and explained the purpose of the study, then informed consent was obtained from them before the withdrawal of blood. The collected blood was then diluted with a ratio of 1:50 using Hank's Balanced Salt Solution (Sigma, St. Louis, MO, USA), followed by the addition of SOZ as an activator. The polymorph nuclear cells (PMS) contained buffy coat layer and mononuclear cells were obtained using dextran sedimentation technique and cells were obtained after LSM (MP Biomedicals, Inc., Germany) density gradient centrifugation. The purified PMNS was collected from the tube as described earlier.[19]

Chemiluminescence assay

The luminol-based assay was performed as described by Helfand, Werkmeister.[20] The luminol was used to measure the level of the released reactive oxygen generated from the whole blood or the isolated PMNs after activation with the opsonized zymosan. Ibuprofen compound was used as a standard control drug for comparison. Luminescence was monitored for 50 min.

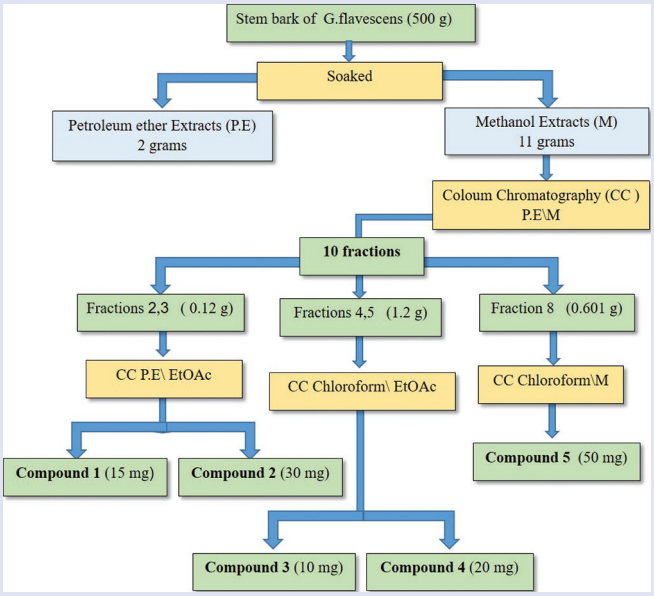

Figure 1: Extraction, fractionation, and isolation of *Grewia flavescence* components

Phytohemagglutinin stimulated T-cell proliferation assay

Peripheral blood mononuclear cells were isolated from heparinized venous blood of healthy adult volunteers by the standard Ficoll-Hypaque gradient centrifugation.[21] The cells were activated, proliferated following the addition of the 1.5 µg/mL PHA (Sigma Chemical Co., USA) as described by Nielsen, Gerwien.[22] The tested compounds were tested at concentrations ranging from 1 to 50 µg/mL and the reaction was incubated for 3 days at 37°C in a CO_2 environment then pulse with tritiated thymidine (0.5 µg/mL) and further incubated overnight to monitor the level of proliferation. Cells were extracted onto a glass fiber filter paper using a cell harvester (SKATRON AS Flow Lab, Norway). The proliferation level indicated by the amount of the incorporated radioactivity was measured using the beta scintillation counter (1211 LKB WALLAC Rack Beta, USA).

Cytotoxicity assay

The MTT assay was performed according to the method of Mosmann[23] and Dimas *et al*, 1998.[24] The Normal 3T3 NIH mouse fibroblast adherent cells and cervical cancer Hela cells were purchased from ATCC. The cells at a concentration of 2×10^5/mL were used for monitoring the cytotoxic properties of serial concentration (0.1–100 µg/mL) of the tested compounds after a 72 h incubation in a CO_2 incubator. The cell suspension was centrifuged for 15 min at 1500 rpm (433 g) at 4°C using AllegraTM X-22R centrifuge (USA). The absorption was measured at 540 nm in a microtiter plate reader (spectrophotometer).

Statistical analysis

All values are expressed as the mean ± standard deviation. All experiments were performed at least three times, each time with three to five independent observations. Statistical analysis was performed by Student's *t*-test to compare the significant mean differences between the control and tested compounds. The differences were considered to be significant at levels of $P \leq 0.05$.

RESULTS

Isolation and characterization of compounds

Five compounds were isolated and identified from the ethanolic extract of *G. flavescens* stem bark. The isolation flow chart is given in Figure 1. The chemical structures of compounds were determined by NMR and mass fragmentation techniques. Compounds 1–3 and 5 were

Table 2: Chemiluminescence effect, PHA- activated T-cell proliferation and cytotoxicity activity for the isolated compounds

Compound	IC50 µg/mL*				
	ROS**generation		T-cellproliferation	Cytotoxicity	
	Whole blood	Neutrophil		Normal cell	Helacancer cell
Lupeol (1)	> 100	> 100	> 50	> 100	23.5 ± 1.7
ß-sitosterol (2)	> 100	> 100	23.6 ± 1.2	> 100	21.5 ± 1.5
Betulin (3)	> 100	> 100	>50	> 100	11.1 ± 0.5
29 (30)-lupene-3, 20-diol;3β-form,29-Aldehyde (4)	> 100	> 100	8.7 ± 0.3	> 100	16.5 ± 0.5
ß-sitosterolglucopyranoside (5)	> 100	> 100	16.7 ± 1.3	> 100	>100
Ibuprofen	11.2 ± 1.9	2.8 ± 0.1	-	-	-
Prednisolone	-	-	0.2 ± 0.3	-	-
Cyclohexamide	-	-	-	0.13 ± 0.02	-

*IC50 values are expressed as mean of three determinations, **ROS; reactive oxygen species

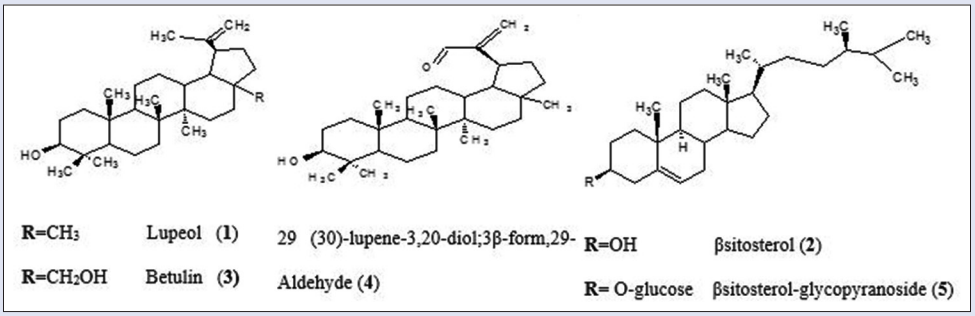

Figure 2: Chemical structure of isolated compounds from *Grewia flavescens* stem bark

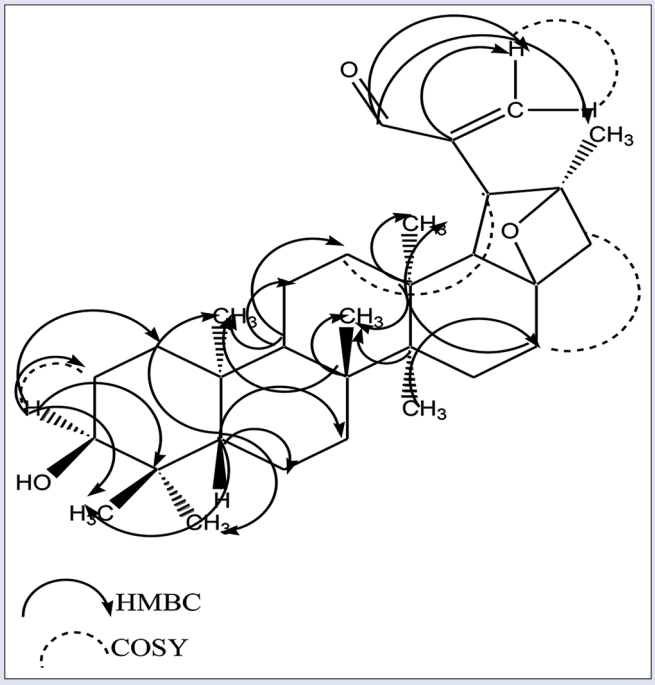

Figure 3: HMBC, COSY 450 & NOESY correlations of compound 4

confirmed by comparison with published literature and authentic specimens and were identified as lupeol (1) (15 mg, 0.14%),[25] ß-sitosterol (2) (30 mg, 0.27%),[26] betulin (3) (15 mg, 0.14%),[27] and ß-sitosterol glucopyranoside (5) (15 mg, 0.14%).[26] [Figure 2].

Compound 4 was obtained as colorless crystals (20 mg, 0.18%) from the $CHCl_3$-MeOH mixture. R*f* value 0.69 in solvent system ($CHCl_3$: MeOH (8:2, v/v)), developed violet color with ceric sulfate. IR spectrum showed absorption at 3334 cm^{-1} (O-H), 2851.9 cm^{-1} (C-H),1735 cm^{-1} (C = O) 1679 cm^{-1} (C = C). EI-MS gave the molecular ion peak at *m/z*: 468 (M+). HREI-MS gave molecular formula $C_{31}H_{48}O_3$. ^{1}H-NMR spectrum (500 MHz, in CD_3OD) showed signals at δ 0.73, 0.79, 0.8, 0.91, 94, 0.99, 1.01 (7 CH_3, each s), 3.17 (1 H, d, 8 Hz), 5.88 (1 H, d, J = 1.4 Hz), 6.26 (1 H, d, J = 3.2 Hz). 2D-NMR: COSY, HMBC, and HMQC spectra are represented in Table 1 and Figure 3. Accordingly, compound 4 was identified as 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde. The spectroscopic data of all isolated compounds have been supplied as Supplementary File 1.

Cytotoxic and immune-modulatory effects

All compounds exerted almost no inhibitory effect (>100 µg/mL) on ROS production on whole blood phagocytes and neutrophils by chemiluminescence assay. The suppressive effect of the isolated compounds on PHA-activated T-cell proliferation stimulated by the thymidine incorporation method showed that 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) significantly repressed cell proliferation (IC_{50} of 8.7 µg/Ml). ß-sitosterol glucopyranoside (5) (IC_{50} of 16.7 µg/mL) and ß-sitosterol (2) (IC_{50} of 23.6 µg/mL) showed moderate activity compared to the standard immunosuppressive drug prednisolone (0.2 µg/mL).

It is of interest to note that none of the tested compounds showed any signs of cytotoxicity in normal cells. On the contrary, a selective cytotoxicity against the cancer cells has been observed. Betulin (3) revealed the highest cytotoxicity activity toward Hela cancer cells, followed by 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) and ß-sitosterol (2) with IC_{50} 11.10, 16.5 and 21.51 µg/ml, respectively [Table 2].

DISCUSSION

Phytochemical study on the ethanolic extract of *G. flavescens* stem bark resulted in the isolation and identification of one new triterpenoid; 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde together with the four known bioactive compounds; lupeol (1), ß-sitosterol (2), betulin (3) and ß-sitosterol glucopyranoside (4). Lupeol (1) is a dietary pentacyclic triterpene found in many vegetables and fruits. It was not toxic[28] and was reported to have potential therapeutic and chemopreventive applications for cancer and inflammation.[28] It was shown to exhibit higher anti-inflammatory activity than indomethacin, a commonly used non-steroidal anti-inflammatory drug in rat and mouse inflammation models. ß-sitosterol is also a well-known dietary phytosterols and has multiple biological properties such as antioxidant, anticancer, antidiabetic, antimicrobial, and immunomodulatory activities.[29] Betulin (3) is reported to retain an antiviral, analgesic, anti-inflammatory, and antineoplastic properties.[30]

Controlling oxidative metabolism by NADPH-oxidase inhibition and ROS scavenger drugs might be of therapeutic value in the treatment of inflammatory and autoimmune diseases.[31] In this study, the immunomodulatory properties of the isolated compounds from *G. flavescens* stem bark were evaluated. Poor chemiluminescence performance was observed for all the isolated compounds, and this could be attributed to the poor electron-donating nature of the aromatic ring substituent, which results in a lower yield of the chemiluminescence-triggering oxidation reaction.[32]

There are two types of immune system, innate and adaptive responses. The adaptive immune response is further classified into humoral and cellular immunity. They are mainly mediated by the T-Lymphocytes, which play a major role in many immune and autoimmune diseases.[32] During chronic inflammation, activated T-cells secrete cytokines, which negatively affect the situation by the activation and proliferation of various other immune cells. Graft

rejection of transplanted organs and tissues is also mediated by the activated T cell through cell-mediated lysis or indirectly by antibody production enhancement or complement activation. Therefore, natural compounds that suppress the activated T cell could be considered as lead immunosuppressive drug.[33] In the present study, the new compound; 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) significantly repressed cell proliferation (IC_{50} of 8.7 µg/Ml). Although both ß-sitosterol glucopyranoside (5) (IC_{50} of 16.7 µg/mL) and ß-sitosterol (2) (IC_{50} of 23.6 µg/mL) exerted moderate effect, it was observed that the glycosylated ß-sitosterol exhibited significantly higher activity that the aglycone form. This observation may be explained, in part, by their physicochemical properties including appropriate lipophilicity.[32] ß-sitosterol has been found to be effective in modulating the secretion of pro/anti-inflammatory cytokines.[34] The immunomodulatory effects of glycoside ß-sitosterol have also been reported earlier.[35] Additionally, clinical trials have been conducted which established the immune-modulatory effects of ß-sitosterol with limited toxic profiles in low doses.[36] However, it has been linked with several toxicity symptoms at high concentrations.[37,38] Together with this finding, our results are in well correlation with the literature showed that both beta-sitosterol and ß-sitosterol glucopyranoside exhibited immune modulation effect.

Many anticancer natural lead compounds fail to participate in the drug development process due to their side effects, which is more than their beneficial effect.[39] As such, any compound which has selective behavior in cancer cells, without harming normal cells will be of high significance. Triterpenoids exhibit remarkable and diverse physiological and therapeutic actions such as antiangiogenic and dedifferentiation effects that are related with anticancer actions.[40] They are even found to be a cancer reversal drug from multidrug resistant cancers.[41-43] It is interesting to note that the anticancer effects of triterpenoids have emerged as a selective apoptosis inducer in breast cancer cells while sparing normal cells.[40] In the present study, betulin followed by the new compound (29 [30]-lupene-3, 20-diol; 3 β-form, 29-Aldehyde) exhibited a significant anticancer effect against the Hela cell line and were proven to be not toxic to normal cell. A previous study had shown that betulin was capable of selective apoptosis induction on invasive breast cancer.[44] In addition, it was found to exhibit potent antitumor potential[45] and apoptosis induction in several other cancer cells, including glioblastoma, leukemia, and lung carcinoma.[46,47] Our results in the current research are in well agreement with the literature indicating that the terpenoids as a phytochemical class with individual phytochemicals having significant anticancer potential.

CONCLUSION

We have isolated a new and four known triterpenoids from the stem bark of *G. flavescens*, which were evaluated for immunomodulatory and cytotoxicity properties. Among all isolated and tested compounds, the new compound; 29 (30)-lupene-3, 20-diol; 3 β-form, 29-Aldehyde (4) significantly interferes with the adaptive immune response (T-cell proliferation). It inhibited the PHA-activated T-cell with an IC_{50} of 8.7 µg/mL. ß-sitosterol and its glycoside form exerted moderate activity. Moreover, betulin and the new compound, respectively, exerted significant anticancer activity against the Hela cell line while ß-sitosterol and lupeol also exhibited considerable anticancer property but with lower effect than the former two compounds.

Taking together all these results, we would like to conclude that *G. flavescens* has a good potential for future studies which might focus in other phytochemicals from the plant and animal model-based studies, which could bring more mechanistic facts about this plant activity.

Acknowledgements

The authors would like to acknowledge the assistance of Dr. Achyut Adhikari of Central Department of Chemistry, Tribhuvan, University, Kirtipur, Kathmandu, Nepal, in the elucidation of compound structures.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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