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Research Article

Cytotoxicity, Wound Healing and Chemical Constituents of Methanol Leaf Extract from *Cassia sieberiana* DC. and *C*assia *singueana* Del. (Fabaceae)

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ABSTRACT

Cassia sieberiana and *C. singueana* are tropical plant species known for their medicinal uses to treat diabetes, ulcer, and other inflammatory related diseases. The cytotoxicity and anti-proliferative activity of the methanol leaf extract of *C. sieberiana and C. singueana* against human embryonic liver (WRL-68), human breast carcinoma cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) were evaluated using MTT assay. The wound healing ability of the extracts was tested by *in vitro* scratch assay using human skin fibroblast (HSF1184) cells. Fractionation and purification of the extracts were carried out in solvents with various polarity using column chromatography techniques. Isolated compounds were characterized by IR, NMR and MS techniques. Both plant extracts were found non-toxic at 250 µg/mL against the liver cell. The extracts does not have any anti-proliferative effect against human breast carcinoma cell lines. However, the extract from *C. singueana* achieved complete cells migration (100.00±0.00%) at 12.5 µg/ml on day 3 against fibroblast cells, while significant proliferation and migration of fibroblast cell (76.17 ± 1.37 to 93.83 ± 0.61%) was observed in dose dependant manner on day 3 for extract from *C. sieberiana*. The extract from *C. sieberiana* afforded the isolation of physcion (**2**), 7-methylphyscion (**3**), islandicin (**4)**, squalene (**10**), while quercetin (**5**), luteolin (**6**), kaempferol (**7**) and dihydrokaempferol (**8**) were obtained from *C. singueana.* Caompound (**1**) and (**7**) demonstrated complete fibroblast cells migration (100.00 ± 0.00%) at 10.00 µM/mL compared to the control L-Ascorbic acid (80.83 ± 0.52%) at same concentration. The cytotoxicity results obtained from this study corroborate the wound healing property observed in the extracts of both plants. The proliferation and migration of fibroblast cells to enhance wound closure could be attributed to the presence of compounds (anthraquinone and flavonoid) isolated from the MeOH leaf extracts of the two *Cassia* species.

Keywords: Cytotoxicity, *Cassia*, Fabaceae, MCF-7, fibroblast, methanol extract

1. INTRODUCTION

Medicinal plants have played an important role in health care and disease management for many years (Romero-daza, 2002). In recent years, the global demand for alternative medicine through primary health care has significantly increased (Chit et al., 2012). Wound is unavoidable throughout human life; about 6 million people suffer from chronic wounds that includes diabetes, ulcer and local infections (Kumar et al., 2007). Chronic wounds may cause multiple organ failure or even lead to death (Roberts et al., 1998). Extracts from plants have demonstrated potentials in the treatment of wounds (Mittal et al., 2016). Hence the need to evaluate the cytotoxicity, wound healing ability and chemical constituents of herbal plants is necessary.

Cassia sieberiana and *Cassia singueana* (Fabaceae) are both shrubs, distributed across Sudano-guinean savanna and widely utilized in traditional medicine (Etuk et al., 2010; Kpegba et al., 2011; Ottu et al., 2013). Decoction of various parts of *C. sieberiana* and *C. singueana* are used to treat inflammatory, skin and venereal diseases (Awomukwu et al., 2015; Etuk et al., 2010: Tamboura et al., 2005). The leaf of *C. sieberiana* is used in the treatment of arthritis, rheumatism, gonorrhoea, toothache, stomach ache, body pain, diabetes, ulcer, malaria, diarrhoea, dysentery, leprosy and liver diseases, (Awah et al., 2012; Tamboura et al., 2005). Similarly, the leaf of *C. singueana* is utilised in the treatment of peptic ulcer, syphilis, malaria, pneumonia, snake bite, diabetes and to enhance blood circulation in nursing mother (Ifeanyi and Ode, 2012; Ode et al., 2011). Previous studies have reported antimicrobial (Asase et al., 2008), antimalarial (Aliyu et al., et al., 2013; Mebrahtom et al., 2014; Saidu et al., 2011), antioxidant (Awah et al., 2012; Ifeanyi and Ode, 2012), hepatoprotective (Gideon et al., 2015; Ode et al., 2011) activities from the leaf extracts of *C. sieberiana* and *C. singueana*. Despite the numerous medicinal uses of the leaf of *C. sieberiana* and *C. singueana*, very few has studied their cytotoxicity, wound healing (Awah et al., 2012; Gideon et al., 2015; Ode et al., 2011; Saidu et al., 2011) and /or chemical constituents (Ode and Asuzu, 2014; Saidu et al., 2019). Cytotoxicity test on plant extracts provides an insight towards the carcinogenic disposition of the plant extract (O'Brien and Haskins, 2006).

In this study, cytotoxic activity of the MeOH leaf extract of *C. sieberiana* and *C. singueana* was tested against human embryonic liver (WRL-68), human breast carcinoma cell line (MCF-7, MDA-MB-231 and MDA-MB-468) using MTT assay.

2. MATERIALS AND METHODS

2.1. Plant materials

The leaf part of *C. singueana* and *C. sieberiana* were collected in January 2016, from Bauchi State, Nigeria. Both plants were identified by Mr. Baha'uddeen Said Adam from the Department of Plant Biology, Bayero University Kano. Voucher specimen, BUKHAN0316 and BUKHAN0065 have been deposited for *C. singueana* and *C. sieberiana* respectively, at the Herbarium of Department of Plant Biology, Bayero University Kano, Nigeria.

2.2. Extraction and purification

The powdered air-dried leaf of *C. singueana* (500 g) and *C. sieberiana* (400 g) were extracted in MeOH (each 3×4 L) at room temperature. The extract was filtered and concentrated under reduced pressure to yield the MeOH leaf extract for *C. singueana* (CALM, 12 g, 2.40%) and *C. sieberiana* (CBLM, 7 g, 1.75%). The MeOH extract from the leaf of *C. sieberiana* (CBLM, 5 g) was subjected to fractionation by VLC eluting with *n*-hexane-EtOAc in an

increasing polarity to afford 4 fractions. Purification of the second fraction (1.8 g) by CC (*n*-hexane: EtOAc; 9:1) gave stigmasterol (**9**) (20 mg) as white solid and chrysophanol (**1**) (15 mg) as yellow solid. The fourth fraction (1 g) was subjected to CC (*n*-hexane: EtOAc; 4:1) to yield physcion (**2**) (18 mg), 7-methylphyscion (**3**) (10 mg) both as orange yellow solids and islandicin (**4**) (12 mg), a reddish solid. Squalene (**10**) (15 mg) was obtained as white solid from repeated CC (*n*-hexane: EtOAc, 3:2) of the fifth fraction (0.8 g). The MeOH extract from leaf of *C. singueana* (CALM, 10 g) was subjected to VLC with *n*-hexane-EtOAc in increasing polarity to afford 5 major fractions. The third fraction (1.2 g) from CALM was purified by CC (*n*-hexane: EtOAc; 9:1) to give stigmasterol (**9**) (23 mg) as a white solid and chrysophanol (**1**) (95 mg) as yellow solid. The fourth fraction (3 g) was subjected to CC and further purified in Sephadex LH-20 (MeOH, 100%) to obtain quercetin (**5**) (55 mg) and kaempferol (**7**) (30 mg) both as yellow solids. The fifth fraction (4 g) was re-fractionated by VLC with *n*-hexane-EtOAc in a polarity gradient manner to give 6 sub-fractions. The third sub-fraction (1 g) was purified by CC and then further purification in Sephadex LH-20 afforded dihydrokaempferol (**8**) (13 mg) and luteolin (**6**) (15 mg) both as yellow solids.

2.3. Cytotoxicity assay

Human normal liver WRL-68, cancer cell lines; HSF1184, MCF-7, MDA-MB-231 and MDA-MB-468 were cultured in 75 cm^2 culture flasks containing RPMI-1640 medium (MCF-7 cells) and Dulbecco modified Eagle's medium (WRL-68, HSF1184, MDA-MB 231 and MDA-MB 468 cells). The culture medium was supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator containing 5% CO_2 in air at 37°C (±1°C). Each cell line was split regularly before attaining 70–80% confluence. Cells were harvested with TrypLE like enzyme and diluted to a suspension in a fresh medium for producing single cell suspensions. Cell Viability: Cells were seeded at a density of 7×10^4 cells/well in 96-well microtiter culture plates. After overnight incubation, the medium was removed and replaced with fresh medium containing different concentrations of crude extracts (0–250µg/ml) diluted from a 5mg/ml stock. After 24 hours of incubation, 10 μL of MTT solution, 5mg/mL in Phosphate-buffered Saline (PBS) was added into each well containing 100 μL medium and incubated at 37˚C for 4 hours. Excessive MTT was discarded and the formazan products made due to dye reduction by viable cells were dissolved using isopropanol containing 0.01 M HCl and the optical density (Abs) was measured with a microplate reader (Biotek Epoch, USA) at a wavelength of 570 nm. The percentage of viability was calculated and compared to a negative control using equation:

$$Viability\ (\%) = 100 - \left(\frac{Abs_{cntrl} - Abs_{treated}}{Abs_{cntrl}} \times 100\%\right)$$

2.4. In vitro scratch assay

The cells ($2x10^5$ cells/mL) in Dulbecco modified Eagle's medium (DMEM) containing 10% FBS were seeded in a 6 well plate. Once the confluent monolayer was formed, a linear scratch was generated in the monolayer with a sterile pipette 200 µL tip. Any cellular debris was removed by washing with phosphate buffer saline (PBS) and replaced with 2 mL of fresh medium in the absence (negative control) and presence of test samples. Wound closure was measured using a fluorescence invert microscope equipped with digital camera (Nikon Eclipse TE200: Nikon, Tokyo, Japan). Images were analysed by NIH image J software by monitoring the width of the scratch area at different time intervals (0, 24, 48 and 72 h) to calculate wound closure. By comparing the images from day 1 to 3, the distance of each scratch closure was determined and the percentage migration rate was calculated. In each well, two scratch were

made (left and right) and per scratch, six points were considered. Average of left scratch and right scratch were taken separately. Percent migration was calculated for left scratch and then right scratch using equation:

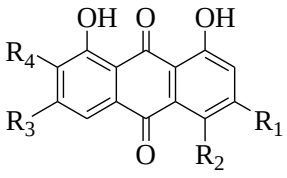
$$Migration\ rate\ (\%) = \frac{Eo - E1}{Eo}$$

where, Eo: average distance between scratch (day 0) and E1: average distance between scratch (day 1 or 2 or 3). Statistical comparisons were estimated by one-way ANOVA followed by Duncan's post hoc test for multiple comparisons with control. Statistical analysis were performed using SPSS 16.0 software. A value of $*p < 0.05$ was considered to indicate statistical significance. All experiments were performed in quadruplicate and the IC_{50} values were expressed as average ±SD.

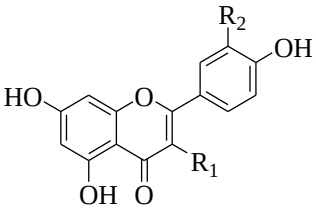
3. RESULTS AND DISCUSSION

3.1. Phytochemical characterization

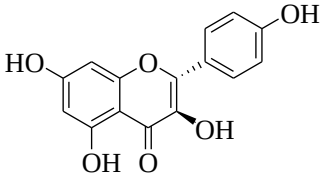
The structure of compounds isolated from the MeOH leaf extract of *C. singueana* and *C. sieberiana* were characterized using various spectroscopic techniques (NMR, IR and MS) and their data compared with literature. Compounds **1**–**4** were identified as the anthraquinones; chrysophanol (**1**) (Sung-kenu et al., 1998), physcion (**2**) (Shen et al., 2012), 7-methylphyscion (**3**) (Sylvain et al., 2010), and islandicin (**4**) (Fiaz et al., 2013). Compound **5**–**8** were elucidated as flavonoids; quercetin (**5**) (Meixian et al., 2011), kaempferol (**7**) (Adebayo et al., 2010), dihydrokaempferol (**8**) (Meixian et al., 2011) and luteolin (**6**) (Mohamed et al., 2015). However, compounds **9** and **10** were triterpenes identified as stigmasterol (Yoo et al., 2006) and squalene (Wei et al., 2010), respectively. Quercetin (**5**), dihydrokaempferol (**8**), kaempferol (**7**) and luteolin (**6**) where obtained from the MeOH leaf extract of *C. singueana*, while physcion (**2**), 7-methylphyscion (**3**), islandicin (**4**) and squalene (**10**) were isolated from *C. sieberiana*. Both plant extracts yielded chrysophanol (**1**) and stigmasterol (**9**).



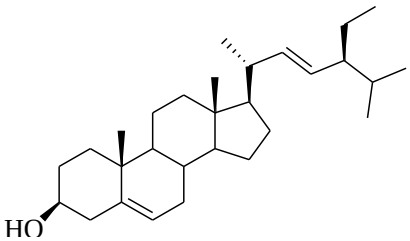
(**1**) $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$
(**2**) $R_1 = CH_3$; $R_2 = R_4 = H$; $R_3 = OCH_3$
(**3**) $R_1 = R_4 = CH_3$; $R_2 = H$; $R_3 = OCH_3$
(**4**) $R_1 = CH_3$; $R_2 = OH$; $R_3 = R_4 = H$



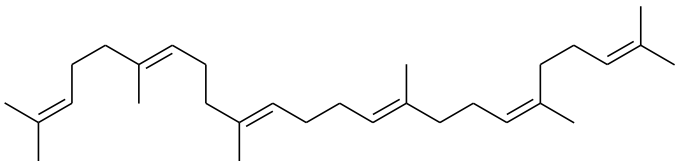
(**5**) $R_1 = R_2 = OH$
(**6**) $R_1 = H$; $R_2 = OH$
(**7**) $R_1 = OH$; $R_2 = H$



(**8**)



(**9**)



(**10**)

Figure 1. Isolated compounds from the MeOH leaf extract of *C. sieberiana* and *C. singueana*

3.2. Cytotoxic activity

The minimal non-toxic dose (MNTD) of each plant extracts against normal liver cells (WRL-68) was determined using MTT assay at range of 0-250 µg/mL. The MNTD for both MeOH leaf extract of *C. singueana* and *C. sieberiana*, was more than 250 µg/mL (Table 1). Thus, the anti-proliferative activity of both plant extracts at range 0-250 µg/mL were tested against breast cancer cells; MCF-7, MDA-MB 231 and MDA-MB 468. From Table 1, it was found that only MeOH leaf extract of *C. singueana* gave IC_{50} of 207±15.72 µg/mL against MDA-MB 231. Therefore, the extracts show no cytotoxic effect on all the cancer cell lines tested based on the United State Cancer Institute (NCI) (Adila et al., 2013).

Similar report of non-cytotoxicity of leaf extract of *C. sieberiana* against human peripheral blood mononuclear cells (PBMC) cell line has been reported (Awah et al., 2012). The anthraquinones; chrysophanol (**1**), physcion (**2**) and islandicin (**4**) were previously reported to have no cytotoxic activity against some cancer cell lines such as KB, MCF-7, MDA-MB 231, K562, A549 and HeLa cells (Lutz et al., 2007; Mai et al 2001; Negera et al., 2014; Victor et al., 2011). Thus, the chrysophanol (**1**) moiety in physcion (**2**), 7-methylphyscion (**3**) and islandicin (**4**) could be responsible for the non-cytotoxic activity of the MeOH leaf extract of *C. sieberiana* against both normal liver cell (WRL-68) and cancer cells (MCF-7, MDA-MB 231 and MDA-MB 468). Although, flavonoids which include quercetin (**5**) was reported to inhibit cell proliferation of cell lines such as MCF-7, MDA-MB 231, HL-60 (Matlas et al., 1994; O'Brien and Gluseppe 2004). However, the possible antagonist effect of chrysophanol (**1**) isolated from the MeOH leaf extract of *C. singueana* could be responsible for the non-cytotoxic effect of the MeOH leaf extract of *C. singueana* observed against the tested cell lines.

Table 1. Cytotoxicity and anti-proliferative assay (IC_{50} values) of tested extracts

Extracts	**Cell lines/IC_{50} (µg/ mL)**			
	WRL-68	**MCF-7**	**MDA-MB 231**	**MDA-MB 468**
CALM	> 250	> 250	207.00 ±15.72	> 250
CBLM	> 250	> 250	> 250	> 250

CALM = Methanol leaf extract for *C. singueana*; CBLM = Methanol leaf extract for *C. sieberiana*

3.3. Scratch assay

The proliferation and migration of fibroblast cells gives good indication of wound healing (Addis et al., 2020). The MeOH leaf extract of *C. singueana* showed significant proliferation of fibroblast cells in a time- and dose-dependent fashion. From Table 2, it is obvious that cells treated with MeOH leaf extract of *C. singueana* at 12.5 µg/mL started to migrate into the denuded area on day 1 (43.05 ± 3.76%) of treatment and scratch closure was completely achieved (100.00 ± 0.00%) on day 3. Quercetin (**5**) and luteolin (**6**) has been reported to increase fibroblast cells proliferation and hence wound healing (Rex et al., 2018; Zahra et al., 2018). The effective scratch closure observed for MeOH leaf extract of *C. singueana* could be attributed to the effect of flavonoids (quercetin, kaempferol, dihydrokaempferol and luteolin) or due to the synergy effect between chrysophanol (**4**) and kaempferol (**7**) isolated from the MeOH leaf extract of *C. singueana*. However, the significant scratch closure ranging from 72.87±3.46% to 93.83±0.61% observed in a dose-dependent manner on day 3 for the MeOH leaf extract of *C. sieberiana* could be due to the concentration of chrysophanol (**1**) in the extract, while the inability to achieve complete scratch closure after day 3 could be attributed to the lack of flavonoid (kaempferol) in the extract.

Plant extracts with phytochemicals such as flavonoids and sterols, that demonstrate significant antioxidant, anti-inflammatory and antimicrobial activity have shown ability to

facilitate wound healing (Gaba and Prasanta 2013; Tumen et al., 2018). Thus, the excellent scratch closure obtained at 12.5 µg/mL on day 3 for MeOH leaf extract of *C. singueana* corroborate the significant antioxidant, antidiabetic and anti-inflammatory activities demonstrated in our previous work (Saidu et al., 2020). The isolation of quercetin (**5**), luteolin (**6**), kaempferol (**7**) and dihydrokaempferol (**8**) from the MeOH leaf extract of *C. singueana* further support the wound healing ability observed for this extract.

Table 2. Effect of crude extracts and isolated compounds on *in vitro* scratch assay using fibroblast HSF1184 cells

Sample	Dose (µg/mL)	% Migration rate of cells		
		Day 1	Day 2	Day 3
CALM	12.5	43.05±3.76	87.07±2.93	100.00±0.00
	25.0	27.80±2.14	82.32±0.79	84.24±0.39
	50.0	6.77±3.18	19.74±2.27	29.86±0.98
	100.0	2.19±0.62	8.92±1.34	9.99±0.77
CBLM	12.5	41.88±1.44	70.15±1.66	72.87±3.46
	25.0	57.24±2.89	76.27±1.37	76.17±1.37
	50.0	37.60±1.62	74.89±1.34	89.40±0.14
	100.0	33.83±2.19	74.68±1.56	93.83±0.61
Quercetin (**5**)	1.25	27.71±3.49	49.25±1.45	60.77±2.17
	5.00	33.47±2.03	47.04±7.50	60.08±2.94
	10.00	39.86±0.45	42.69±1.38	70.70±1.46
Physcion (**2**)	1.25	9.60±1.54	23.88±0.92	29.83±0.80
	5.00	32.42±2.53	45.97±1.75	53.80±3.04
	10.00	34.68±3.79	51.70±2.56	55.45±1.63
Kaempferol (**7**)	1.25	37.32±3.47	66.95±0.15	72.56±3.73
	5.00	47.33±1.96	76.88±1.52	78.35±1.20
	10.00	57.27±1.82	92.88±0.58	100.00±0.00
Chrysophanol	1.25	49.22±2.52	100.00±0.00	100.00±0.00
	5.00	38.66±3.27	73.32±2.00	93.23±1.22
	10.00	62.01±0.20	100.00±0.07	100.00±0.00
Dihydrokaempferol (**8**)	1.25	35.72±1.08	69.69±0.86	75.81±0.45
	5.00	26.16±1.40	45.68±1.49	54.66±0.75
	10.00	36.95±3.21	46.64±1.10	50.11±2.064
L-Ascorbic	1.25	22.81±5.03	54.61±1.57	74.53±1.43
	5.00	31.71±1.08	55.96±0.87	75.79±2.57
	10.00	36.65±2.13	68.27±3.33	80.83±0.52
Control	-	37.26±1.11	52.40±1.98	72.88±0.61

CALM = Methanol leaf extract for *C. singueana*; CBLM = Methanol leaf extract for *C. sieberiana*

For the isolated compounds, chrysophanol (**1**) and kaempferol (**7**) demonstrated complete cells migration (100.00 ± 0.00%) at 10.00 µM/mL compared to the control *L*-Ascorbic acid (80.83±0.52%) at same concentration. Cells treated with chrysophanol (**1**) achieved complete cells migration on day 2 of treatment and scratch closure was hence complete. The possible protection of the OH groups (through hydrogen bond with C=O) on the ring of chrysophanol (**1**) could be responsible for the better fibroblast cell proliferation observed for this compound, chrysophanol (**1**) (Lutz et al., 2007).

4. CONCLUSION

The MeOH leaf extracts of *C. singueana* and *C. sieberiana* were non-cytotoxic (IC_{50} > 20 µg/mL) to the tested cell lines (WRL-68, MCF-7, MDA-MB 231, and MDA-MB 468) but

rather stimulated the proliferation and migration of fibroblast HSF1184 cells. These effects could be attributed to the anthraquinone compounds (chrysophanol, physcion, 7-methylphyscion and islandicin) isolated from the MeOH leaf extract of *C. sieberiana* and/or the flavonoids (quercetin, kaempferol, dihydrokaempferol and luteolin) isolated from the MeOH leaf extract of *C. singueana*. The results obtained from this study demonstrates that MeOH leaf extract of both *Cassia sieberiana* and *C. singueana* has cytoprotective effects and wound healing properties, as such, these extracts might not cause harm to humans within the tested dose limits. Thus, the popular use of the leaf of *C. sieberiana* and *C. singueana* in the treatment of diabetes, ulcer, skin disorder, and other wound related ailments (Asha et al., 2023) could be attributed to the presence of those compounds isolated from these plants.

Conflict of Interest
The authors declare that there is no conflict of interest.

Author Contribution Statement
Saidu Jibril: Conceptualization, Methodology, Writing - Original Draft, Writing - Reviewing and Editing. Sayang Baba.: Investigation, Writing - Review & Editing. Aminu Muhammad: Writing - Reviewing and Editing. Hasnah Mohd Sirat: Resources, & Supervision. Salehhuddin Hamdan: Resources, Writing - Reviewing and Editing. Norazah Basar: Investigation, Formal Analysis, Resources & Supervision. Adoum Oumar Al-Moubarak: Writing - Reviewing and Editing.

Data Availability Statement
The authors confirm that the data supporting the findings of this study are available within the article.

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