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Research Article

QUANTIFICATION OF QUERCETIN AND STIGMASTEROL OF *COUROUPITA GUIANENSIS* AUBL BY HPTLC METHOD AND *IN-VITRO* CYTOTOTOXIC ACTIVITY BY MTT ASSAY OF THE METHANOL EXTRACT AGAINST HeLa, NIH 3T3 AND HepG2 CANCER CELL LINES

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ABSTRACT

The methanol extract of the dried flower of *Couroupita guianensis* was subjected to the HPTLC analysis. Further the extract was screened for cytotoxicity against the HeLa, NIH 3T3 and HepG2 cancer cell lines. It showed significant cytotoxicity with IC_{50} values of 202.6, 280 and 470.8 µg/ml towards HeLa, NIH 3T3 and HepG2 cancer cell lines respectively. Two compounds stigmasterol (I) and quercetin (II) were isolated from the methanol extract and identified by their IR, NMR and Mass spectral data. Further I and II were quantified by HPTLC methods (242.14 µg/ml and 188.97 µg/ml respectively). II was identified as the biomarker compound.

Keywords: *Couroupita guianensis,* Cytotoxicity and HPTLC.

INTRODUCTION

Couroupita guianensis Abul belongs to family *Lecythidaceae* was first described [1, 2, 3]. It is used extensively as an ingredient in many preparations which cure gastritis, scabies, bleeding piles, dysentery, scorpion poison and many. It has rube facint and anti rheumatic properties used in ayurvedic concepts, cold relief balm and fruit pulp is used to cure headache, the flowers are used to cure cold, intestinal gas formation and stomach ache, and also for treating diarrhoea, and when dried and powdered, used as a snuff [4]. The fragrance of flowers is used for curing asthma and the shell of the fruit is used as a utensil and also the free radical scavenging activity and HPLC was carried out [4], the flowers of *C. guianensis* showed analgesic and anti-inflammatory [5] and immunomodulatory activity [6]. Petroleum ether and chloroform extracts of this plant exhibited larvicidal activity against vectors [7]. *Couroupita guianensis* leaves are rich in phytochemicals and reported to contain quercetin, saponins and tryptanthrin [8]. The tree is also rich in providing anthocyanin, flavanoids, volatile constituents like eugenol and farsenol. From the flowers of *Couroupita guianensis* stigmasterol and aliphatic hydrocarbon have been isolated [9]. So far, no study on the *in-vitro* cytotoxic activity of methanolic extract of these flowers has been recorded. Therefore, the main objectives of the present study are to determine the *in-vitro* cytotoxic activity of methanolic extract of the dry flowers of *C. guianensis,* and also isolation and identification of the phytoconstituents and quantification of the main phytoconstituents in the methanolic extract of *C. guianensis* flowers.

MATERIALS AND METHODS

Plant material

Fresh flowers of *C. guianensis* was collected in February, 2010, from Palakkad district, Kerala and the plant species was authenticated in the Department of life science, Karpagam University, Coimbatore-21. Voucher specimen was preserved in our Department (No. KU11CHE1934).

Extraction

The dried flower (300 g) of C. *guianensis* (sample CGA) was extracted with methanol (500 ml) using soxhelet type apparatus. Then the methanol extract was concentrated, and weighed (753 mg). The residue was used for column chromatography.

HPTLC analysis

50 mg of the methanol extract was weighed accurately in an electronic balance (Afcoset) and dissolved in 1ml of methanol and centrifuged at 3000 rpm for 3 minutes and this homogeneous solution was used as test solution for HPTLC analysis.

Isolation and characterization of quercetin (II) and stigmasterol (I)

Methanol extract of dried *C. guianensis* flowers was subjected to coloumn chromatography. The column was packed with 60 g of silica gel and 600 mg of the extract was loaded in to the column. And a mixture of petroleum ether and ethyl acetate is used as a solvent system (8:2) and monitored by TLC. Concentration of the like fractions (12-20) yielded stigmasterol (I) and was characterized by IR, ^{13}C-NMR, ^{1}H-NMR and MS spectral data.

The IR ν_{max} (KBr) cm^{-1}; 3373 (O-H); 2940 and 2867 (C-H stretching); 1641 (C=C); 1457.3 (CH_2); 1381 and 1038 (cycloalkane).

^{1}H-NMR (CDCl3, 400 MHz): δ 0.79, 0.78, 0.82, 0.91, 0.94, 1.08 (m, 18H), δ 1.13 to δ 2.53 (m, 18H, 9x CH_2 and 8H), 3.54 (1H, dd, J= 9.1, 6.0 Hz, H-α3), 5.33 (s, 1H, H-3), 5.11 and 5.15 (2H, br);

^{13}C-NMR (CDCl3, 400 MHz): δ 33.79 (C-1), 78.98 (C-3), δ 145.83 (C-5), δ 121.81 (C-6), δ 32.50 (C-7), δ 35.35 (C-8), δ 45.99 (C-9), δ 30.02 (C-14), δ 56.93 (C-17), δ 19.91 (C-19), δ 138.39 (C-22), δ129.35 (C-23), δ 30.35 (C-24), δ 18.89 (C-28);

FAB-MS *m/z* (rel.int): 412 [M^+] ($C_{29}H_{48}O$) (100), 397 (20), 369 (10), 351 (70), 329 (65), 300 (40), 299 (23), 273 (31), 255 (28).

Concentration of the fractions 68-80 eluted with ethyl acetate and methanol (80:20) and when monitored by TLC showed a mixture of three compounds with Rf value 0.65, 0.69 and 0.73 (major compound). The fractions were combined and concentrated to yield 68 mg of the mixture 1.

Quercetin (II) was isolated from the mixture 1 by using preparative TLC plate with the mobile phase Toluene-Acetone-Formic acid (4.5: 4.5: 1), and was characterized by IR, ^{13}C-NMR, ^{1}H-NMR and MS. spectral data.

The IR ν_{max} (KBr) cm^{-1}; 3397.96 (-OH), 2919.70(C-H), 1621.84 (C=C), 1462.74 (CH_2).

^{1}H-NMR (DMSO, 400 MHz): δ 6.18 and 6.40 (both d, J=1.7Hz, H-6 and 8), δ 7.68 (d, J=2.5Hz, H-2'), δ 6.89 (d, J=8.3 Hz, H- 5'), δ 7.53 (dd, J=8.6Hz, 2.2 Hz, H-6'), δ 12.42 (s, 5(OH)).

^{13}C-NMR (DMSO, 400 MHz): δ 147.5 (C-2), δ 136.4 (C-3), δ 176.5 (C-4), δ 161.4 (C-5), δ 98.9 (C-6), δ 164.5 (C-7), δ 94.0 (C-8), δ 156.8 (C-9), δ 103.7 (C-10), δ 122.6 (C-1'), δ 115.8 (C-2'), δ 145.7 (C-3'), δ 148.4 (C-4'), δ 115.5 (C-5'), δ 119.9 (C-6');

FAB-MS *m/z* (rel.int): 348 [M^+] $C_{15}H_{10}O_7$ (100), 302(98), 257(15), 228(8), 201(8), 154(8), 136(9), 110(9), 70(4) and 23(3).

Standard preparation

The given standard stigmasterol (I), and quercetin (II) weighed (1mg) separately in an electronic balance (Afcoset) and dissolved in 1ml of methanol and this solution was used as a standard solution for HPTLC analysis.

Sample application

1µl of test solution and 2µl of standard solution were loaded as 5mm band length in the 3 x 10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The sample loaded plate was kept and developed in TLC twin trough developing chamber (after saturated with Solvent vapour) with respective mobile phase, Toluene-Acetone-Formic acid (4.5: 4.5: 1) and petroleum ether 60°C-80°C - Ethyl acetate (8: 2)) for quercetin (II) and stigmasterol (I).

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254nm and UV366nm.

Derivatization

The developed plate was sprayed with respective spray reagent (20% Sodium carbonate reagent and brief dried followed by Folin Cio-calteu reagent and Anisaldehyde sulphuric acid reagent (quercetin and stigmasterol) and dried at 100° C in Hot air oven. The plate was photo-documented in Day light mode using and UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV light 254nm. The Peak table, Peak display and Peak densitogram were noted.

Analysis details

Mobile phase

Toluene-Acetone-Formic acid (4.5: 4.5: 1) (quercetin) (II)

Petroleum ether 60°C-80°C - Ethyl acetate (8: 2) (stigmasterol) (I)

Spraying reagent

20% Sodium carbonate reagent and brief dried followed by Folin Cio-calteu reagent and Anisaldehyde sulphuric acid reagent; for quercetin (II) and stigmasterol (I) respectively.

***In -vitro* anti cancer activity**

The human cervical cancer cell line (HeLa), Human laryngeal epithelial carcinoma cells (HepG2), Hepatocellular carcinoma cells and (NIH 3T3) mouse embryonic fibroblasts were obtained from National Centre for Cell Science (NCCS), Pune. The HeLa and HepG2 cells were grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and NIH 3T3 fibroblasts were grown in Dulbeccos Modified Eagles Medium (DMEM) containing with 10% FBS. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/ml. One hundred micro liters per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the extracts and fractions. They were initially dissolved in neat dimethylsulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred micro liters per well of each concentration was added to plates to obtain final concentrations of 100, 50, 25, 12.5 and 6.25 µM. The final volume in each well was 200 µl and the plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 48h. The medium containing without samples were served as control. Triplicate was maintained for all concentrations [10, 11, 12].

MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

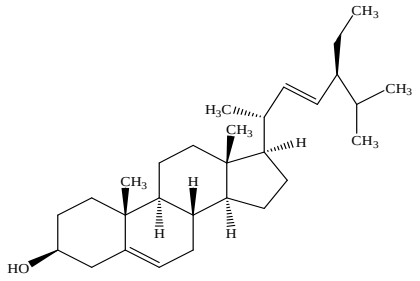
Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using Graph Pad Prism software.

RESULT AND DISCUSSION

Isolation and characterization

Stigmasterol (I)

The FAB-MS spectrum showed a molecular ion peak at *m/z* 412 corresponding to molecular formula $C_{29}H_{48}O$. The IR spectrum exhibited strong absorptions at 3422 cm^{-1} (hydroxyl group), 2951 and 1669 cm^{-1}. The ^{1}H-NMR spectrum exhibited six tertiary methyl groups at δ 0.79, 0.78, 0.82, 0.91, 0.94, and 1.08, one vinylic proton at δ 5.33 and two olefinic protons at δ 5.11 and 5.15 respectively. In ^{13}C-NMR spectrum the most down field signals at δ 145.83 was accommodated for sp^2 (olefinic) carbon at C-5 and the next downfield signal at δ 138.39 ppm and δ 129.35 ppm to C-22 and C-23. The downfield signal at δ 121.81 is to C-6. The oxygenated carbon at C-3 gave a downfield signal at δ 78.98 ppm. The next downfield signal at δ 56.93 ppm was accommodated for C-17. Other carbon atoms of the steroidal skeleton except that in the side chain appeared in the range δ 45.99 to δ 30.02 ppm. The angular methyl groups and the side chain methyl carbons gave signals in the region δ 19.91 to δ 18.89 ppm. These spectral data resembled those described in literature [13].

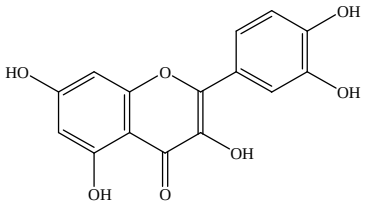


I

Quercetin (II)

The FAB-MS spectrum showed a molecular ion peak at *m/z* 348 corresponding to molecular formula $C_{15}H_{10}O_7$. The IR spectrum exhibited strong absorptions at 3397.96 cm^{-1} (hydroxyl group), 1621.84 cm^{-1} (C=C group), 2919.70 and 1462.74 cm^{-1}. The ^{1}H-NMR δ (DMSO): 6.18(1H, d, J=1.7Hz), 6.40(1H, d, J=1.7Hz) are due to meta-coupled protons of A-ring (H-6 and H-8) of a flavonoid nucleus. Signals at δ = 6.89 d= 8.3Hz, 7.68d, 2.5Hz and δ = 7.55dd, 2.2Hz, 8.3Hz are assigned to H-5', H-2' and H-6' of the ring. The 1H NMR spectrum showed protons at aromatic regions from 6- 8 ppm, and strong hydrogen bonding at 12.5ppm. These suggest a quercetin nucleus. The ^{13}C-NMR spectra revealed 15 carbon signals typical of

flavonoid monoglycoside nucleus. The low field signal at 176.5ppm was due to the carbonyl group at C-4 [14, 15].



II

HPTLC analysis

The table 1 and 2 and fig 1, 2 and 3 and 4 indicate the presence of quercetin (II) (Standard Rf-0.72 cm and extract-0.73 cm) and stigmasterol (I) (Standard Rf- 0.42cm and extract- 0.42cm) in methanol extract of *C. guianensis*. The corresponding chromatograms also clearly confirm the presence of quercetin (II) and stigmasterol (I) in methanol extract of *C. guianensis* flowers. Blue colored zones at daylight mode were observed in the tracks of the chromatogram after derivatization, which confirmed the presence of quercetin (II) in the standard and in the sample. In the other plate blue, violet and pink colored zones at day light mode were present in the tracks of the chromatogram after derivatization, which confirmed the presence of stigmasterol (I) in the given standard and in the sample. Table 1, and Fig 1 and 3 indicate that the extract exhibited spots for 10 compounds out of which three were phenolic compounds including the quercetin (II) (Rf-0.72). Further from the area of the spots it indicates that the quantity of quercetin (II) was more when compared with other compounds. It can be considered as a biomarker for *C. guianensis*. In Table 2 and fig 2 and 4 it indicates the presence of atleast nine steroids including stigmasterol (I) at an Rf value 0.42. Both the compounds quercetin (II) and stigmasterol (I) were quantified and the quantities are 242.14 µg/ml and 188.97 µg/ml respectively. So far to the best of our knowledge compounds were isolated and identified but was not quantified in the whole plant of *C. guianensis*. This is the first time quercetin (II) and stigmasterol (I) were quantified in the methanolic extract of *C. guianensis* flowers.

Table 1: TLC data of the methanolic extract of the dried flower of *C. guianensis*

Track	Peak	Rf	Height	Area	Assigned substance
Sample CGA	1	0.13	106.7	2504.1	Unknown
Sample CGA	2	0.17	24.2	225.4	Phenolic 1
Sample CGA	3	0.21	26.2	834.8	Unknown
Sample CGA	4	0.30	29.3	1298.1	Phenolic 2
Sample CGA	5	0.41	11.9	137.4	Unknown
Sample CGA	6	0.45	10.2	177.4	Unknown
Sample CGA	7	0.57	117.7	4883.2	Phenolic 3
Sample CGA	8	0.73	283.8	18316.5	Quercetin(II)
Sample CGA	9	0.87	65.4	2910.8	Unknown
Sample CGA	10	0.96	226.3	8990.9	Unknown
Que	1	0.72	655.1	14372.4	Quercetin(II) standard

Table 2: TLC data of the methanolic extract of the dried flower of *C. guianensis*

Track	Peak	Rf	Height	Area	Assigned substance
SGL	1	0.42	94.5	3447.4	Stigmasterol(I) standard
Sample CGA	1	0.08	20.3	270.3	Steroid 1
Sample CGA	2	0.18	20.1	412.0	Steroid 2
Sample CGA	3	0.36	22.7	795.8	Unknown
Sample CGA	4	0.42	89.4	3428.7	Stigmasterol(I)
Sample CGA	5	0.48	34.8	917.8	Steroid 4
Sample CGA	6	0.69	97.3	5054.9	Steroid 5
Sample CGA	7	0.83	67.1	2518.5	Steroid 6
Sample CGA	8	0.90	71.9	2512.1	Steroid 7
Sample CGA	9	0.95	91.7	2239.2	Steroid 8

Fig. 1: (day light)
Solvent system
(Toluene-Acetone-Formic acid (4.5: 4.5: 1)

Fig. 2: (UV 366 nm)
solvent system
(Petroleum ether 60°C-80°C - Ethyl acetate (8: 2)

Fig. 1&2: Quercetin (II) and stigmasterol (I) from TLC profile of the methanol extract of CGA after derivatization

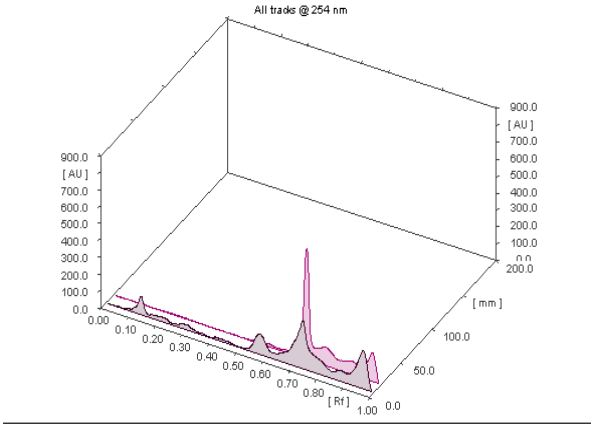


Fig. 3: Pink colour peak indicate the standard track of quercetin (II)

Brown colour indicate the methanolic extract of sample CGA

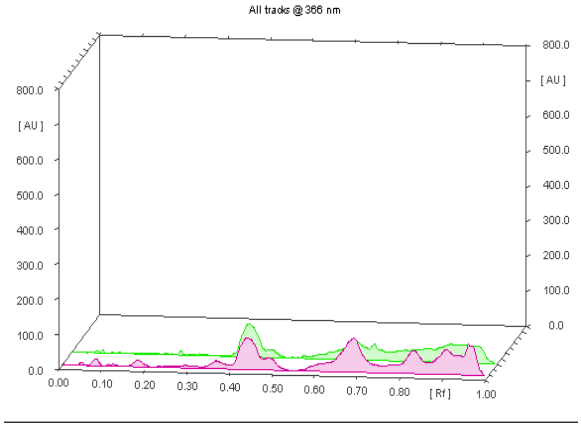


Fig. 4: Green colour indicate the standard track of stigmasterol (I)

Pink colour indicate the methanolic extract of sample CGA

Fig. 3&4: Quercetin (II) and stigmasterol (I) from HPTLC chromatogram

MTT assay

We employed MTT assay, a simple and reliable technique, which measures cell viability for screening the *in-vitro* anticancer activity and the results are shown in table 3. The viability of cancer cells after incubation with different concentrations of *C. guianensis* flowers methanol extract are depicted in Fig.5. (HeLa cell line), Fig.6. (NIH 3T3 cell line) and Fig.7. (HepG2 cell line). The incubation with different concentration of methanol extract (1.75, 2.1, 2.45, 2.60 and 3.0 µg/ml.) affected the viability of human cervical cancer cell line (HeLa) and hepatocellular carcinoma carcinoma cells (HepG2) and mouse embryonic fibroplasts (NIH 3T3). The methanol extract of *C. guianensis* dried flowers showed cytotoxic effect on the HeLa, HepG2 and NIH 3T3 cancer cell lines in dose dependant pattern and the IC_{50} values were determined as 202.6, 280 and 470.8 µg/ml, respectively. This is the first report of its kind to test the methanol extract of *C. guianensis* for anticancer activity. The methanol extract was more active towards HeLa cell lines, when compared with HepG2 and NIH 3T3 cell lines.

Table 3: *In vitro* cytotoxicity activity against from methanol extracts of *C. guianensis* against NIH 3T3/ HeLa/ HepG2 cancer cell lines.

Name of the cell lines	IC_{50} µg/ml
HeLa	202.6
NIH 3T3	280
HepG2	470.8

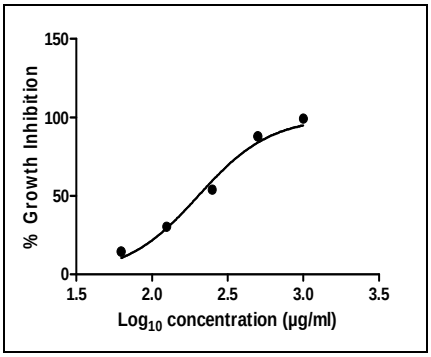


Fig. 5: MTT assay of *C. guianensis* on HeLa cell line

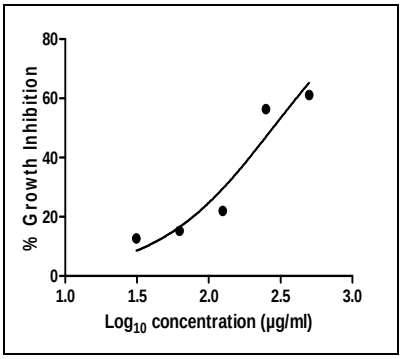


Fig. 6: MTT assay of *C. guianensis* on NIH 3T3 cell line

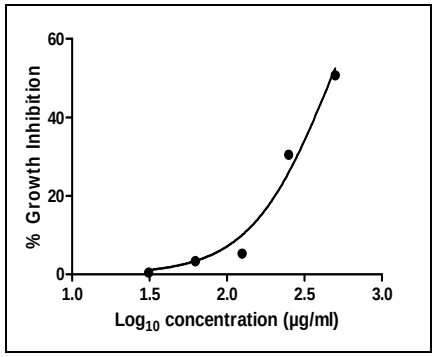


Fig. 7: MTT assay of *C. guianensis* on HepG2 cell line

CONCLUSION

The methanolic extract of *Couroupita guianensis* showed *in-vitro* cytotoxic activity against HeLa, HepG2 and NIH 3T3 cancer cell lines by MTT assay. Two compounds stigmasterol (I) quercetin (II) (biomarker) and were isolated and identified by spectral methods. Further the isolated compounds were quantified by HPTLC method.

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