JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. **7**(4), p. 2707-2712

In vitro Antimicrobial, Antioxidant and Cytotoxic Activities of New Pregnane Glycosides and Pregnanes Isolated from the *Carallum adescendens* var. *gracilis* and *Caralluma pauciflora*

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(Received: 19 January 2013; accepted: 03 March 2013)

Since ages plants and their products are an inseparable part of mankind in the treatment of the various diseases as they are a good source of different types of chemical constituents and Secondary metabolites. Medicinal uses of *Caralluma* species have been reported in the treatment of cancer, inflammatory diseases, diabetes, tuberculosis skin rashes, leprosy, and diseases of blood, snake and scorpion bites. The present study deals with the in-vitro anti microbial, anti fungal and antioxidant Activities of one pregnane (CPG-I) glycoside and two pregnanes (CP-I&CP-II) from *Caralluma pauciflora (Asclepiadaceae)*. The compounds were tested against bacteria and fungi to determine the Antimicrobial and anti fungal activity by the micro broth dilution method. In-vitro antioxidant activity of these compounds was evaluated using DPPH free radical scavenging, H_2O_2 and reducing power methods. The steroidal glycoside (CPG-I) and pregananes (CP-I&CP-II) showed good antimicrobial, anti fungal and anti-oxidative activity. The results indicate that plant derived compounds could be an alternative to the current available pharmaceutical drugs in the market.

Keywords: Broth dilution, free radical scavenging activity, cytotoxic, *Caralluma adscendens* var gracillis, *Caralluma pauciflora*.

Carallumas succulents beloning to Asclepiadaceae family; these have both edible and medicinal uses[1-7]. Hot water extract of stem of *Caralluma* edulis is used as remedy for diabetes[8-10] *Caralluma Umbelleta* claimed to be useful in treating stomach disorders and abdominal pains[11]. According to traditional practioners of Africa *Carallumas* are useful in treating rheumatism, diabetes, leprosy and as antiseptics and disinfectants[12]. Bedionus use Caralluma negevensis in treating chronic lung diseases such as tuberculosis and cancer[13].

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In continuation of earlier studies on Indian *Carallumas* here we report the biological activity of three compounds which were reported by one of us from *Caralluma adscendens* var. gracilis (CPG-I) and *Caralluma pauciflora* (CP-I&CP-II)[14].

Caralluma pauciflora (Asclepiadaceae) (Wright) N.E. Brown is a perennial herb growing wild in the states of Tamil Nadu and Karnataka, India. Morphologically it resembles C.indica. It contains fewer (2-3) flowers (as umbels) than C. indica (8-10 flowers).During the flowering season only they can be distinguished otherwise it is very difficult. The genus Caralluma is a rich source of pregnane glycosides. In recent years, several pregnane glycosides with interesting biological activity have been reported from this genus[14-18].

Caralluma adscendens var. gracilis (Gravelyet Mayaranathan) grows wild in Tamilnadu (Pudukottai, Salem and Ramnad districts) and Karnataka states of India. It grows to a height of 60 cm and has angled stems. It bears pink, hairy flowers in pairs all along the stem[6]. The genus Caralluma is a rich source of pregnae glycosides. In recent years, several pregnane glycosides with interesting biological activity have been reported from this genus[14-18].

MATERIALS AND METHODS

Materials

Microbial cultures, MTS solution (Promega), 96 well flat bottom plates, Amphotericin-B, Neomycin 2, 2'-diphenly-1-picrylhydrazyl (DPPH), H_2O_2, methanol mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$] (2.5mL, 1%) and test compounds (CPG-I), (CP-I&CP-II).

Plant material

Caralluma adscendens.var.gracillis and *Caralluma pauciflora* (Asclepiadaceae) were collected from the Satyamangalam village of Pudukotai district, Tamil Nadu, India. The plants were identified and authenticated by Prof V.S. Raju, Department of Botany, Kakatiya University, Warangal, Andhrapradesh, India. The extraction, isolation and structural elucidation of these compounds were already reported[14].

Microbial strains

Five bacteria of both gram positive and gram negative organisms such as, Escherichia coli (ATCC35218), Staphylococcus aureus (ATCC 43300), Entero faecalis (ATCC 5129), Klebsialla pneumoniae (ATCC700603), Pseudomonas aeruginosa (ATCC 27853) and four yeast strains of Candida from American Type Culture Collection out of which two are Candida albicans strains (Candida albicans ATCC 90028, Candida albicans ATCC 10231), and two are non-albicans Candida stains (Candida krusei ATCC 6258, Candida parapsilosis (ATCC 22019) were used in the present study.

Antibacterial susceptibility test

The bacterial susceptibility test was carried out using the micro broth dilution method dissolving test compounds in DMSO[19].The cultures were adjusted to a turbidity of 0.5 McFarland standards after 16-18 h of incubation at 37 °C. The working suspension of 106 CFU mL^{-1} at 625 nm was obtained by diluting a stock suspension of 0.4-5×108 CFU $mL^{-1}$to hundred fold. Microtiter plates were placed in laminar flow unit for aseptic conditions. Aliquots of 100LL of bacteria inocula were added to the microtiter plates containing different concentrations of test compounds which were incubated aerobically at 37°C for 24 hrs, 40 LL of freshly prepared iodo nitro tetrazolium chloride2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H tetrazolium chloride, INT) solution (200µg mL^{-1}) was added to each well and the plates were further incubated for 45 minutes at 37°C in the dark. Reduction of INT to red after incubation indicates persistent growth of bacteria; no color change indicates lack of bacterial growth, all analyses were performed in triplicates and data is reported.

Antifungal susceptibility test

The susceptibility of Candida albicans and non- Candida albicans species was evaluated using the micro broth dilution method according to M27-A2 for yeast guidelines[20]. Yeast strains were grown aerobically at 35 °C on Sabouraud dextrose agar plates for overnight on Yeasts were harvested and suspended in a 1% sterile saline and the turbidity of the supernatant was measured using spectrophotometer at 625 nm with an absorbance of 0.08-0.1 equivalents to the 0.5 McFarland standards following the NCCLS M27-A2 guidelines. The working suspension was diluted 1:20 in a mixture containing RPMI 1640 medium with 0.165M morpholine propanesulfonic acid buffered to pH 7.0. The working suspension was further diluted with the medium (1:50) to obtain the final test inocula (1-5x103CFU mL^{-1}). The micro titer plates were allowed to thaw and equilibrate to room temperature under aseptic conditions which contains different concentrations of test compounds. Aliquots of working inocula suspensions were dispensed into each well and the plates incubated in an aerobic environment at 35°C for 24h. After incubation, 20 Ll of 3- (4,5-dimethylthiazol-2-yl) -5 - (3-carboxymethoxyphenyl) -2 - (4-18sulfophenyl) -2H- terazolium salt (MTS, Promega Corporation, Madison, USA) was added directly to each well, incubated at 37 °C for 4 h and the absorbance recorded at 490 nm on a 96-well plate reader

(VACUTEC). All analyses were performed in triplicate and the data is ,reported as the mean ± standard error of ≤ 5.

***In vitro antioxidant* activity**

DPPH radical scavenging activity

Free radical scavenging activity was determined by using 2, 2'-diphenly-1-picrylhydrazyl (DPPH) method followed by Burits and Bucar (2000). One ml of various concentrations of the CPG I, CP I and CP II in methanol were added to 4ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. Inhibition of free radical by DPPH in percent (I %) was calculated by using the following equation.

$$I\ \% = [(A\ control - A\ sample) / A\ blank] \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test Compound), and A sample is the absorbance of the test compound.

H_2O_2 scavenging activity

The H_2O_2 scavenging activity of both plant extracts was determined according the standard method[21]. A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of CPG I, CP I and CP II in 3.4 ml phosphate buffer were added to a H_2O_2 solution (0.6mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The % of inhibition was calculated.

Reducing power

The reducing power was determined according to the Oyaizu (1986) method; Ascorbic acid was used as a standard. Different concentrations of tested compounds were prepared in methanol and mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1%). The mixture was incubated at 50°C for 20 min and 2.5ml of trichloroaceticacid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

RESULTS

The present study was aimed to determine the biological activity of novel pregnane glycoside (CPGI) and pregnanes(CP-I&CP-II) isolated from *Caralluma adscendens*.Var.gracillis and *Caralluma pauciflora* Species. These compounds were tested for antibacterial and antifungal activities.

Antibacterial activity

All of these compounds exhibited good antibacterial. The results are shown in the Table 1; Pregnane glycoside (CPGI) exhibited good antibacterial activity on S.aureus at 12.5µg ml^{-1} concentration whereas pregnanes (CPI&CPII) exhibited in the range of 25-100µg ml^{-1} concentration on the bacteria used in the present study.

Antifungal activity

Compared with antibacterial these compounds showed a good antifungal activity on Candida albicans and non- Candida albicans species ranging from 12.5-100µg ml^{-1} concentrations. The antifungal activities of these compounds were shown in the Table 2.

Antioxidative activity

The new compounds isolated from Caralluma were evaluated for in vitro antioxidant Properties by employing DPPH free radical scavenging and H_2O_2 activity. Increase in the Concentrations of the compounds there is an increase in the % of free

Table 1. Results of Minimum Inhibitory Concentration (MIC µgrams ml-1) of steroidal on various

	Bacterial Strains				
	S.aureues	*E.faecalis*	*E.coli*	*P.aeruginosa*	*K.pneumoniae*
1	12.5	50	50	50	100
2	25	25	62.5	62.5	50
3	12.5	12.5	50	62.5	100
C	10	20	5	10	5

Table 2. Results of Minimum Inhibitory Concentration (MIC µgrams ml^{-1}) of steroidal glycosides on Various Yeast strains

S.	Fungal Strains			
No	*C.albicans*	*C.albicans 10231*	*C.krusei*	*C.parapsilosis*
1	12.5	12.5	62.5	50
2	12.5	12.5	50	50
3	50	50	12.5	100
C	0.125	0.5	0.75	0.75

Table 3. Reducing power activity of CPG I, CP I and CP II compounds All experiments were carried out in triplicate; ascorbic acid was used as a control

	50	100	150	200
CPG I	0.423	0.535	0.679	0.770
CP I	0.395	0.496	0.576	0.698
CP II	0.456	0.576	0.659	0.745
Ascorbic acid	0.479	0.559	0.695	0.786

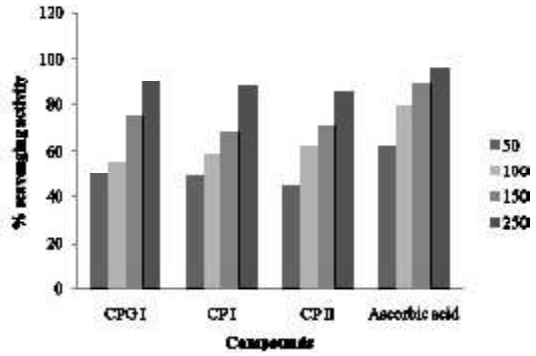

Fig. 1. Free radical scavenging activity of CPG I, CP I and CP II compounds using DPPH. Ascorbic acid was used as a control., all experiments were carried out in triplicate

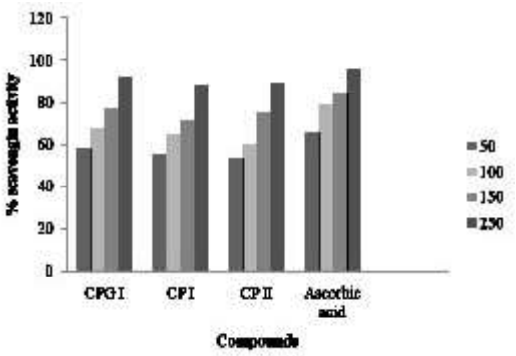

Fig. 2. Radical scavenging activity of CPG I, CP I and CP II compounds using hydrogen peroxide (H_2O_2).Ascorbic acid was used as a control, all experiments were carried out in triplicate

I, CP I and CP II increased with the dose-dependent antioxidant activity. Increased absorbance with increased concentrations showed the increased reducing power activity Table 3. At 200µg ml^{-1} concentration the compounds CPG I and CP II exhibited good reducing power activity with Similar to ascorbic acid which is used as a control. The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity[22].

DISCUSSIONS

Steroidal glycosides exhibit various radical scavenging activity. The results of in vitro antioxidant property of pregnanes glycosides and pregnanes are shown in Fig.1 & Fig.2. At 250µg ml^{-1}, the pregnanes glycoside (CPG I) exhibited 90 % scavenging activity followed by CP I (88 %) followed by CP II (86%) by DPPH method. By H2O2 method, the order of scavenging activity was as follows; CPG I (92 %) followed by CP II (89%) followed by CP I(88%). In both methods Ascorbic acid was used as control. Reducing power of CPG bioactivities including Anti bacterial, anti fungal and anticancer. While the lipophilicity of glycoside moiety influences mainly the pharmacokinetic properties, the steroid moiety exhibit the diverse biological actions via different functional groups located around the periphery of its rigid tetra cyclic core. The paper describes the antimicrobial, antioxidant and cytotoxic activities of new pregnanes glycoside and pregnanes isolated from the whole plant extracts of *C. adscendens Var.gracillis* and *C. pauciflora*. Data obtained from the results showed that they possess good in-vitro antimicrobial, and antioxidant activities.

All the compounds in the present study showed good antibacterial, antifungal and anti oxidative activity compared with the control drugs, neomycin for antibacterial, Amphotericin-B for antifungal and ascorbic acid for anitioxidative activity (Table 1 & Table 2 and Fig 1&2).

The test compounds showed good activity on gram negative organisms than gram positive organism, our studies showed good promising antifungal and antibacterial activity than the earlier reports of steroidal glycosides isolated from the leaves of Solenostemma argel exhibited weak antibacterial and antifungal activities[23-25]. The order of anti bacteria and anti fungal activity is as follows CPG I>CPI≥CPII.

Antioxidants play vital role in inhibiting and scavenging radicals, thus providing protection to Humans against infection and degenerative disorders. Antioxidants are compounds that help to Inhibit many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, Hydroxyl radicals, thereby preventing or delaying damage to the cells and tissues[26].

In all of the anti oxidative investigations namely DPPH free radical scavenging and H_2O_2 activity carried in the present study CPGI showed good activity followed by CPI and CPII, the good this is mainly because presence five different sugar moieties attached at C 3, hydroxyl groups around the steroidal skeleton and benzoyl groups at C-12 and C-20 in CPGI.

Overall In our present study compound CPGI exhibited good antibacterial, antifungal and anti oxidative activities than CPI&CPII this may because of presence of five different sugar moieties linked at C-3 and two benzoyl groups linked at C-12 and C-20 positions[27]. Presence of sugars at C-3 increases the cell proliferation of the compound and aromaticity of two benzoyl groups increase the biological action of the compound, the order of activity is as follows CPGI>CPGI≥CPGII.

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