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Kaempferol-*7*-*O*-Glucoside and their Antimicrobial Screening isolate from *Cassia renigera* Wall.

D. Singh[1], S. K. Sharma[1], Rachana Rani[2*], Sudeep Mishra[2], R. A. Sharma[1]

[1]*University of Rajasthan, Jaipur-302 055, Rajasthan, India*
[2]*National Institute of Science Communication and Information Resources, Dr. K. S. Krishnan Marg, New Delhi- 110 012, India*

ABSTRACT
Cassia species have been of keen interest in phytochemical and pharmacological research due to their excellent medicinal values. Different classes of natural products, possessing potent physiological and pharmacological activities have been isolated from *Cassia renigera*. They include anthracene derivatives, flavonoids and polysaccharides. Some of these compounds have been shown to possess considerable antimicrobial activity. The present study deals with the isolation, purification of flavonoids in different parts of *C. renigera* and their antimicrobial activity. A new flavonoid kaempferol-7-*O*-glucoside was identified and characterized and it showed significant antimicrobial activities. The higher level of total flavonoids was measured in flowers of *C. renigera* (1.15 mg/gdw) and, Similarly higher levels of total qurecetin (F+B) were measured in flowers of *C. renigera* (0.34 mg/gdw). Higher levels of total kaempferol (F+B) were measured in flowers of *C. renigera* (0.60 mg/gdw). Similarly higher levels of kaempferol-7-*O*-glucoside were measured in flowers of *C. renigera* (0.21 mg/gdw). The isolated flavonoids were effective against all test bacteria and fungi but the quercetin was found more effective against *Escherichia coli, Aspergillus flavus, Aspergillus niger, Fusarium moniliformae and Rhizoctonia bataticola,* therefore the MIC value 2×10^3 mg/disc was recorded and MIC value for other bacteria and fungi was recorded as 3×10^3 mg/disc. Kaempferol was more effective against *A. flavus, A. niger, F. moniliformae and R. bataticola,* therefore the MIC value 2×10^3 mg/disc was recorded. MIC value for kaempferol-7-*O*-glucoside (a new flavonoid) was 2×10^3 mg/disc for *E. coli, A. flavus* and *A. niger* but MIC value for *S. aureus, P. aeurinosa* and *S. typhi* was recorded 3×10^3 mg/disc.

Keywords: Cassia renigera, Kaempferol, Quercetin, Kaempferol-7-*O*-glucoside, IR, NMR and Flowers.

INTRODUCTION

Since the advent of modern drug treatments, traditional medicine has greatly receded in occidental societies. Moreover, only a limited number of medicinal plants have received detailed scientific scrutiny thereby prompting the World Health Organisation to recommend that this area be comprehensively investigated. [1] *Cassia renigera* Lamk. is used extensively in various parts of the world against a wide range of ailments, the synergistic action of its metabolite production being most probably responsible for the plant's beneficial effects. [2-3] Plants used as source of indigenous medicines due presence of various secondary metabolites. In the present investigation, the flavonoids with a new flavonoid were isolated and their antimicrobial activities were carried out. Among natural phenolics, the flavonoid forms the largest group and more than 2,000 flavonoids are reported among woody and non-woody plants. [4-7]

***Corresponding author: Ms. Rachana Rani,** National Institute of Science Communication and Information Resources, Dr. K. S. Krishnan Marg, New Delhi- 110 012, India; **E-mail:** rachana.chemistry@gmail.com

A number of plants have been screened for the presence of flavonoids, as reported by many researchers. [8-11] Thus traditional medicinal plants derived antioxidants may protect against a number of diseases and reduce oxidation processes in food systems. [12-13] In order to establish this, it is imperative to measure the markers of baseline oxidative stress particularly in human health and disease and examine how they are affected by supplementation with pure compounds or complex plant extracts from the traditional medicinal plants. [14] The data so far generated clearly sets the basis for a clear understanding of the phytochemistry of the plant and derived cultures and opens the possibility of the potential utilization of the phenolic rich extracts from medicinal plants in food system or as prophylactics in nutritional/food supplement programs. [13, 15] The flavonoids have more importance in providing strength and resistance to the plant against various diseases but also exhibit various biological and pharmacological activities. [16-19] A survey of literature suggested that the flavonoids are found both, in free and bound forms, the former being in higher levels than the later. [20] In the present study a new flavonoid kampferol-7-O-glucoside with the other flavonoids were isolated by

chromatography and characterized them by spectral studies and antimicrobial activity of these isolated compounds was carried out by following the established protocols.

MATERIAL METHODS

Collection and Identification

Cassia renigera Lamk. is a weed of rainy season which is found usually in the shade of trees, crevices of rocks and also in the open gravelly substratum, often hidden amongst grasses. Plant species collected from Jaygragh fort of Ajmer, and Garganesh temple of Jaipur, Rajasthan, India. The plant was identified at Herbarium, Department of Botany, University of Rajasthan, Jaipur, and their voucher specimen (No. 3024) has been deposited in the Harbarium.

Processing and Extraction

The plant parts (root, stem, leaves, flowers and pods) of *C. renigera* were studied for flavonoids composition. Plant sample was extracted with 80% methanol [21] and the concentrated extract was fractionated with petroleum ether (Fraction I), diethyl ether (Fraction II) and ethyl acetate (Fraction III), respectively. Fr. I was rejected in each case due to rich in fatty components whereas; Fr. II and Fr. III were analysed for free and bound flavonoids respectively. Later, Fr. III was hydrolyzed by acid (7% H_2SO_4; 10 ml/g) and re-extracted with ethyl acetate (Fr. IV), followed by neutralization. [9] Thin layer chromatography of Fr. II and Fr. IV were done along with the standard markers[7]. All the solvents were used of analytical grade from Merck (India). The TLC Aluminum sheet, 60 F_{254} (20×10 cm) (Cat. No. 1.05554.0007) was purchased from E. Merck (Mumbai). A number of solvent tried for good resolution but better resolution has been found solvent system (benzene: acetic acid: water: 125: 72: 3). The developed chromatograms were viewed under UV light alone and in the presence of ammonia fumes and subsequently sprayed with the characteristic reagents. [22]

Fluorescent spots of the samples coinciding with the standard. Bands are matched with the standard at kaempferol (R_f 0.85), quercetin (R_f 0.78), and kaempferol-7-*O*-glucoside (R_f 0.81). These samples were isolated by TLC; then it is eluted and purified. Later, the isolated compounds were crystallized and identified by using melting point, UV, IR and NMR spectroscopy. [23] Using spectrophotometric methods of the quantification was made of kaempferol, quercetin and kaempferol-7-*O*-glucoside, respectively. [6, 24]

Test Microorganisms

Standard strains of *Staphylococcus aureus, Salmonella typhyi, Escherichia coli and Pseudomonas aeruginosa* were obtain from microbiology Laboratory SMS medical college, Jaipur, Rajasthan and *Aspergillus flavus, Aspergillus niger, Fusarium monilliformae and Rhizoctonia bataticola* were obtain from seed pathology laboratory, Department of Botany, University of Rajasthan, Jaipur, India.

Antimicrobial Screening

The Disc Diffusion method was used to determine the antimicrobial activities of the isolated flavonoid using standard procedure [25] of 6 mm disc were prepared from whatman's filter paper no. 1. Solutions of varying concentrations ranging from 1.0×10^4 to 5.0×10^4 mg/ml/disc were prepared. Isolated flavonoids were also prepared using the pure extracting solvent for each extract. Nutrient agar was prepared, sterilized and used as the growth medium for the culture of microorganisms; 20 ml of the sterilized medium was poured into each sterilized petri dish, covered and allowed to solidify. The treated discs were air dried at room temperature, to remove any residual solvent which might interfere with the determination, sterilized and inoculated. These plates were initially placed at low temperature for 1 hour so as to allow the maximum diffusion of compounds from the test disc into the agar plate and later incubated at 37°C for 24 hours in case bacteria and 48 hours for fungi, after which the zone of inhibition could be easily observed five replicates of each text extract were examined and the mean values were then referred.

RESULT AND DISCUSSION

Structure Elucidation of Isolated Flavonoids

In the present investigation, flavonoids profile has been studied *in-vivo* of *C. renigera*, where kaempferol-7-*O*-glucoside, kaempferol and quercetin from different plant parts of *C. renigera* have been evaluated by chromatographic, spectroscopic and color reactions.

The compounds eluted from TLC were pooled together according to their TLC behaviour and isolate them with the suitable solvents and evaporated yielding three flavonoids kaempferol-7-*O*-glucoside, kaempferol and quercetin. [19] The spectral analyses of the active constituent, kaempferol-7-*O*-glucoside; kaempferol and quercetin from the different plant parts of selected *C. renigera* are shown below: -

Kaempferol-7-*O*-glucoside: brownish needles on crystallization (m.p. 317°-329°C)

UV light absorption MeOH: 235 sh, 240 sh, 259 sh, 374 sh, 424 sh;

IR: νcm^{-1}/ max KBr: 3600 (glycoside), 3420 (O–H), 1700 (C=O), 1600, 1610, 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815;

^{1}HNMR (300MHz, $CDCl_3$): 5.1 (H_1), 6.68 (H_2), 7.64 (H_3), 6.04 (H_4), 6.03 (H_5), 5.20 (H_6), 6.81 (H_7), 7.16 (H_8), 6.70 (H_9), 5.91 (H_{10}), 3.91 (H_{11}), 2.37 (H_{12}), 3.40 (H_{13}), 2.48 (H_{14}), 3.76 (H_{15}), 2.41 (H_{16}), 3.49 (H_{17}), 2.31 (H_{18});

^{13}C NMR (300MHz, $CDCl_3$): 70.6 (C_1), 75.3 (C_2), 78.8 (C_3), 92.4 (C_4), 154.8 (C_5), 154.2 (C_6), 114.6 (C_7), 137.5 (C_8), 124.0 (C_9), 136.0 (C_{10}), 121.1 (C_{11}), 149.4 (C_{12}), 97.5 (C_{13}), 123.1 (C_{14}), 129.0 (C_{15}).

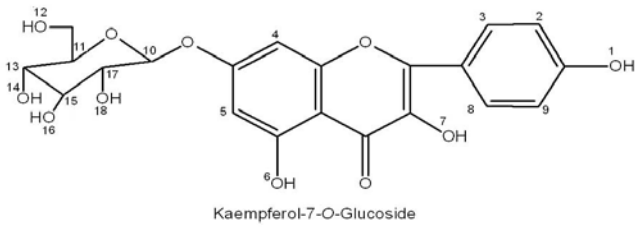

Fig. 1: Structure of Kaempferol-7-*O*-glucoside

Kaempferol: brownish needles on crystallization (m.p. 312°-313 °C)

UV light absorption MeOH: 253 sh, 269 sh, 305 sh, 374 sh, 424 sh;

IR: νcm^{-1}/ max KBr: 3420 (O–H), 2830 (C-H), 1700 (C=O), 1600, 1610, 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815;

^{1}HNMR (300MHz, $CDCl_3$): 2.35(H_1), 7.01(H_2), 7.18 (H_3), 6.29 (H_4), 6.37 (H_5), 2.35 (H_6), 5.39 (H_7), 5.36 (H_8), 7.18 (H_9), 7.01 (H_{10});

^{13}C NMR (300MHz, $CDCl_3$): 1.36 (C_1), 129.8 (C_2), 126.8 (C_3), 131.9 (C_4), 147.4 (C_5), 154.2 (C_6), 114.6 (C_7), 137.5

(C_8), 124.0 (C_9), 136.0 (C_{10}), 121.1 (C_{11}), 149.4 (C_{12}), 106.9 (C_{13}), 131.9 (C_{14}), 126.1 (C_{15}).

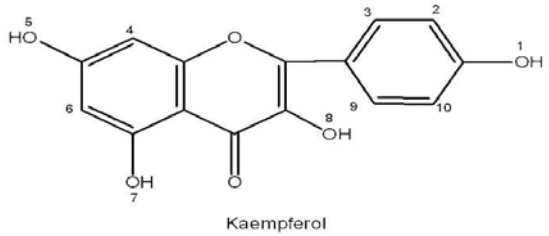

Fig. 2: Structure of Kaempferol

Quercetin: yellowish needles on crystallization (m.p. 312°-313 °C)

UV light absorption MeOH: 255 sh, 301 sh, 374 sh, 440 sh;

IR: νcm^{-1}/ max KBr: 3420, 3380(O–H), 2800 (C-H), 1680 (C=O), 1610, 1610, 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010;

^{1}HNMR (300MHz, $CDCl_3$): 2.45, (H_1), 2.55 (H_2), 6.79 (H_3), 6.98 (H_4), 6.49 (H_5), 2.33 (H_6), 6.38 (H_7), 2.36 (H_8), 5.37 (H_9), 1.4 (H_{10});

^{13}C NMR (300MHz, $CDCl_3$): 137.3 (C_1), 137.9 (C_2), 14.2 (C_3), 127.0 (C_4), 126.1 (C_5), 133.8 (C_6), 142.4 (C_7), 158.2 (C_8), 114.6(C_9), 134.5 (C_{10}), 123.0 (C_{11}), 138.0 (C_{12}), 121.1 (C_{13}), 149.4 (C_{14}), 108.9 (C_{15}), 127.8.

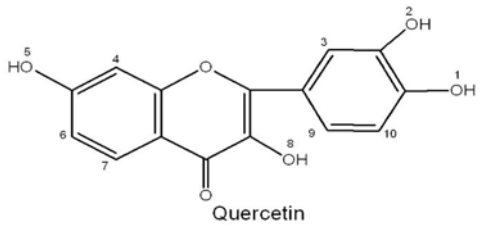

Fig. 3: Structure of Quercetin

Concentration of isolated flavonoids

The results of the chromatographic data and concentration of isolated flavonoids contents (mg/gdw) are summarized in Table 1 and 2 respectively. The isolated flavonoids were identified on the basis of color reactions UV light, I_2 vapours, spraying with chromogenic reagents and by the R_f values as mentioned in Table 1.

Although both free and bound flavonoids were isolated from all the plant part but the bound form of newly identified flavonoid- kaempferol-7-*O*-glucoside was found absent in roots, stem and pods. The maximum concentration of both free and bound flavanoids was observed in the flowers (free = 0.86 mg/gdw, bound = 0.29 mg/gdw). The minimum concentration of both free and bound flavonoids was observed in roots i.e. 0.11 mg/gdw and 0.02mg/gdw, respectively. Among the isolated flavonoids, kaempferol was recorded in more concentration in flower (0.46 mg/gdw, free form) than the isolated other flavonoids. In the bound form, kaempferol was also recorded higher concentration in flowers (0.14 mg/gdw). Over all in the whole plant the isolated free flavonoids were recorded in higher concentration (1.3 mg/gdw) than the bound flavonoids (0.95 mg/gdw).

Antimicrobial Activity

Histochemically plant have provided good source of anti-infective agents which are highly effective against the microbes. The present study was aimed at determining the phytochemistry and antimicrobial activities of isolated flavonoids against pathogenic bacteria and fungi Table 3 and Fig. 4.

The results of antibacterial activity showed that all the isolated flavonoids were effective against all test bacteria but quercetin was recorded more effective against *E. coli* (Inhibitory Zone = 26 mm) while kaempferol-7-*O*-glucoside was very less effective against *P. aeuroginosa* (IZ = 7 mm) but it is more effective against the *S. typhi* (IZ = 12 mm) in comparison to other isolated flavanoids.

The antifungal activity was carried out against the test fungi. All the test fungi were found sensitive to the isolate flavonoids but again quercetin was found more effective against *A. flavus* (IZ = 24 mm) and *A. niger* (IZ = 24 mm) and minimum inhibition activity was showed by kaempferol against *R. bataticola* (IZ = 6 mm) and *F. moniliformae* (IZ = 6 mm).

Table 4 shows the results of MIC for isolated flavonoids for against test microorganisms recorded in mg/disc of the diametrical sections of the respective zones of inhibition for each metabolite. The quercetin was higher effective against *E. coli, A. flavus, A. niger, F. moniliformae and R. bataticola* therefore the MIC value 2×10^3 mg/disc was recorded and MIC value for other bacteria and fungi 3×10^3 mg/disc was recorded.

Table 1: Chromatographic data and colour reaction of the flavonoids isolated from *C. renigera*.

Flavonoids (aglycones)	R_f						Colours by chromatogenic sprays colour			
	BeAW$^+$	BAW*	TBA^{++}	Day-light	UV* ammonia	I_2 vapours	$FeCl_3$		$AlCl_3$	
							Visible	UV	Visible	UV
Kaempferol-7-O-glucoside	0.81	0.78	0.49	YW	BN	BN	BN	BW	YW	YW
Kaempferol	0.85	0.83	0.55	GN-YW	BT-YW	YW-BN	BN	BK	YW	YW-GN
Quercetin	0.78	0.64	0.41	GN-YW	YW	YW-BN	BT-GN	BK	DL-YW	YW-GN

Abbreviations : $^+$BeAW = Benzene : Acetic acid : Water (125 : 72 : 3); BK = Black; BN = Brown; BT = bright
*BAW = n-Butanol : Acetic acid : Water : (4: 1:5); DL = dull; GN = green; YW = yellow
$^{++}$TBA = t-Butanol : Acetic acid : Water (3:1:1)

Table 2: Isolated flavonoid content (mg/gdw*) in the *Cassia renigera*.

C. renigera part		Free (F)				Bound (B)				Total (F+B)			
		Quercetin	Kaempferol	Kaempferol-7-*O*-glucoside	Total	Quercetin	Kaempferol	Kaempferol-7-*O*-glucoside	Total	Quercetin	Kaempferol	Kaempferol-7-*O*-glucoside	Total
1.	Root	0.02	0.07	0.02	0.11	0.02	0.00	0.00	0.02	0.04	0.07	0.02	0.13
2.	Stem	0.04	0.08	0.03	0.15	0.04	0.02	0.00	0.06	0.08	0.10	0.03	0.21
3.	Leaves	0.10	0.25	0.04	0.39	0.09	0.04	0.02	0.15	0.19	0.29	0.06	0.54
4.	Flowers	0.24	0.46	0.16	0.86	0.10	0.14	0.05	0.29	0.34	0.60	0.21	1.15
5.	Pods	0.13	0.16	0.04	0.33	0.06	0.03	0.00	0.09	0.19	0.19	0.04	0.42
6.	Total	0.53	1.02	0.29	1.84	0.31	0.23	0.07	0.59	0.84	1.25	0.36	2.45

Table 3: Bactericidal and fungicidal efficacy of isolated flavonoids from *Cassia renigera*

Test organism		Flavonoids		
		Kaempferol	Quercetin	Kaempferol-7-*O*-glucoside
A. Bacteria				
E. coli	IZ*	15.00	26.00	17.00
	AI*	0.62	1.02	0.68
S. aureus	IZ*	12.00	11.00	15.00
	AI*	0.50	0.45	0.62
P. aeruginosa	IZ*	11.0	12.00	7.00
	AI*	0.41	0.46	0.28
S. typhi	IZ*	9.00	11.00	12.00
	AI*	0.31	0.45	0.50
B. Fungi				
A. flavus	IZ*	22.00	24.00	16.00
	AI*	0.85	0.74	0.80
A.niger	IZ*	21.00	24.00	9.00
	AI*	0.85	0.74	0.56
R. bataticola	IZ*	6.00	18.00	21.00
	AI*	0.20	0.73	0.84
F. moniliforme	IZ*	6.00	8.00	16.00
	AI*	0.33	0.38	0.76

(+) Trace activity; (-) Not measurable activity; Standard : Zentamycin = 10□g/disc

*IZ = Inhibition zone (in mm) including the diameter of disc (6 mm)

$$\text{Activity index} = \frac{\text{Inhibition area of the test sample}}{\text{Inhibition area of the Standard}}$$

Bacteria

A = *E. coli* B = *S. aureus*

C = *P. aeruginosa* D = *S. typhi*

Fungi

E = *A. flavus* F = *A. niger*

G = R. *bataticola* H = *F. moniliforme*

1 = Kaempferol

2 = Quercetin

3 = Kaempferol-7-*O*-glucoside

S = Standard gentamycin for bacteria

S = Standard mycostatin for fungi

Kaempferol was more active only *A. flavus, A. niger, F. moniliformae and R. bataticola* therefore the MIC value 2×10^3 mg/disc was recorded. Kaempferol-7-*O*-glucoside shows MIC value 2×10^3 mg/disc for *E. coli, A. flavus* and *A. niger* but MIC value for *S. aureus, P. aeurinosa* and *S. typhi* 3×10^3 mg/disc was recorded.

In general, work on the flavonoid, in *Cassia* species has centred on the chemical aspects and accordingly a number of glycosides have been characterized, viz.: Kaempferol-3-*O*-□-D-monopyranosyl from *C. grantis* and *C. auriculata* [26, 27]; 3, 5, 3′, 4′, 5′-pentahydroxy-7-methoxyflavone-8-c-l-rhamnopyranoside from *C. sophera* [28];

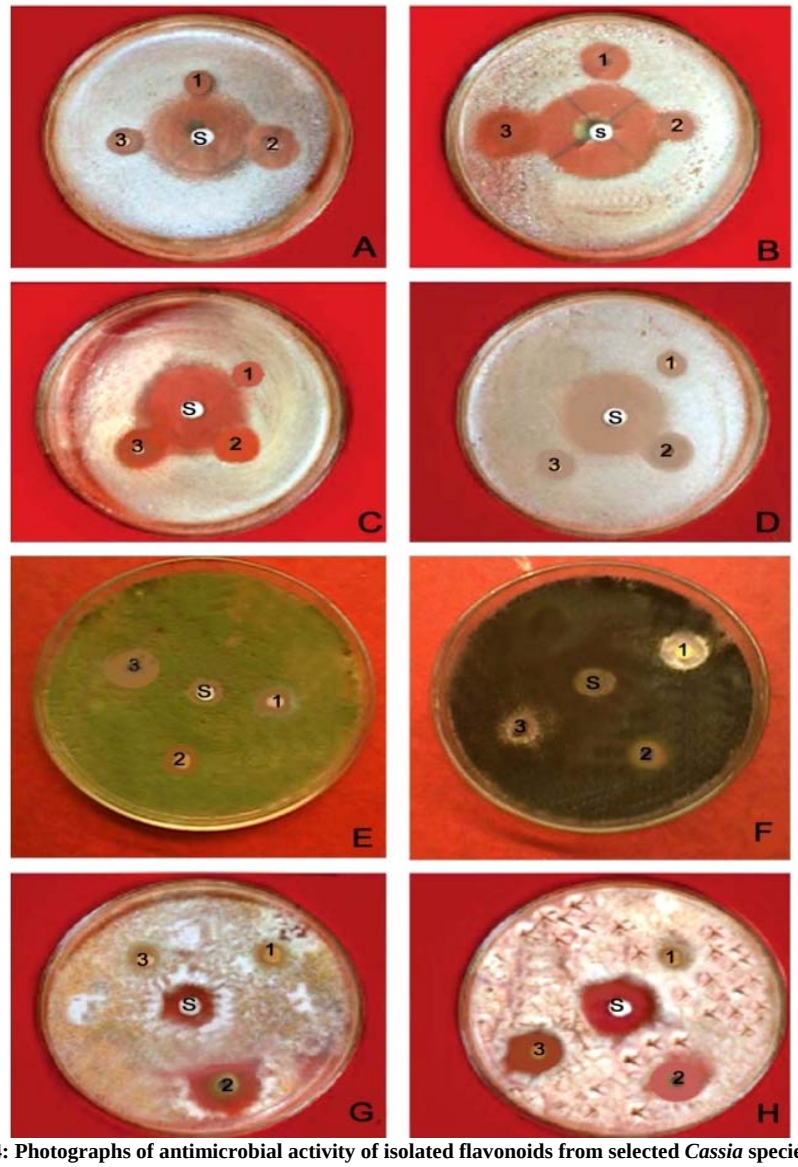

Fig. 4: Photographs of antimicrobial activity of isolated flavonoids from selected *Cassia* species.

Table 4: Zones of inhibition of different concentration (MIC*) of isolated flavonoids (mg/ml).

Test microorganism	Quercetin					Kaempferol					Kaempferol- 7-*O*- glucoside				
	1×10^3	2×10^3	3×10^3	4×10^3	5×10^3	1×10^3	2×10^3	3×10^3	4×10^3	5×10^3	1×10^3	2×10^3	3×10^3	4×10^3	5×10^3
Bacteria															
E. coli	-	+	+	+	+	-	-	-	±	+	-	+	+	+	+
S. aureus	-	-	-	+	+	-	-	-	±	+	-	-	+	+	+
P. aeurginosa	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
S. typhi	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
Fungi															
A. flavus	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
A. niger	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
R.. bataticola	-	±	+	+	+	-	-	±	±	+	-	-	±	+	+
F. moniliformae	-	-	+	+	+	-		±	±	+	-	-	±	+	+

*MIC = Minimum inhibitory concentration

Velutin (5,4′-tihydroxy-7,3′-dimethoxy flavone from *C. renigera* [29]; 5,7,3,5′-tetrahydroxy-6,8, dimethoxy flavone-3-*o*-∝-arobinopyranosite and 5,7,4′-trihydroxy-6,8,3′-trimethoxy flavone-3-*O*-∝-L-rhamnosyl (1□2)-*O*-β-D-gluco-pyranoside from *C. fistula* [29-30]; apigenin 6-c-β-D-olioside from *C. torosa* [31] and some aglycones- kaempferol, quercetin and myricetin from *C. biflora* [22, 32-33], Kaempferol-7-methylether from *C. javanica.* [35]

Some species of the genus *Cassia* exhibited pathological-physiological and biological-activities, their chemistry has been extensively studied, incidentally, very less attention has been paid to study the flavonoid pattern in general, and the *Cassia* species, in particular, but very few reports have been published on the production of flavonoids and their antimicrobial activities in *Cassia* species. [26-27, 29, 32-34]

CONCLUSION

On the basis of above results, it is concluded that the *C. renigera* accumulates certain secondary metabolites in different parts which shows various antimicrobial and pharmacological activities. Therefore, it is a potential source of indigenous medicine to cure various ailments.

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