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QUALITATIVE ANALYSIS OF PHYTOSTEROL AS CHEMOTAXONOMIC MARKERS AND ANTIOXIDANT ACTIVITY OF SOME CUCURBITACEAE SEEDS

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ABSTRACT

Momordica charantia, Trichosanthus dioica, Coccina grandis, Lagenaria siceraria, Cucurbita maxima were evaluated for identification and separation of phytosterols using Thin Layer chromatography and Paired Affinity Index methods. The Solkowaski test and Liebermann Burchard's Test were performed to identify the phytosterols while Paired Affinity Index was used to calculate and correlate the chemical affinities between all the selected texa. In study of TLC Benzene: ethyl acetate (5:1) solvent system showed violet blue colored bands (Rf 0.29) similar to reference standard β-sitosterol. It is a simple, precise method for identification and estimation of phytosterols in seeds. As phytosterols have other advantageous health benefit mainly reducing blood cholesterol in animal and human as well, these seeds of Cucurbitaceae containing plant sterols also shows Antioxidant activity.

KEYWORDS: Phytosterols, Thin Layer chromatography, Paired Affinity Index, Antioxidant activity.

INTRODUCTION

Plant derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-recourse of drugs in traditional system of medicines, but in addition to this, they have the potential of nutraceutical, food supplements, folk medicines pharmaceutical intermediates and chemical entities for synthetic drugs. Plants thus contain an enormous number of biologically active compounds with various chemical structures. These phytochemicals, often secondary metabolites present in higher plants include the alkaloids, flavonoids, tannins, terpenoids, sterols, and many others. Out of these,

Sterols present in many plants as an imperative phytoconstituent. During the last 10 years, there has been an unprecedented intensification of interest in phytosterol. Sterols present in plants known as phytosterols while present in animals known as zoosterol or cholesterol.

Momordica charantia, Trichosanthus dioica, Coccina grandis, Lagenaria siceraria, Cucurbita maxima are species of flowering plants in the squash family known by the common names Bitter gourd, Pointed gourd, Scarlet gourd, Long melon, Pumpkin respectively.[1] Species of cucurbits are distributed in most countries of the world, especially in the tropics and they are now cultivated in every country, state and province where crop plants can be grown in the summer (warm temperature).[2]

During the last decades a large number of cucurbitacins have been isolated from various plant species belonging to the family Cucurbitaceae. The fruits of plant belong to these Cucurbitaceae have been used as folk medicines in some countries because of their wide spectrum of pharmacological activities such as anti-inflammatory and anticancer effects.[3]

The fruits of cucurbits are very useful in terms of human health i.e, purification of blood, remedy on constipation, good for digestion and give energy. Seeds, flowers are also consumed by humans. Seeds, fruits and some parts of cucurbits are reported to possess purgative, emetics and antihelmintics properties due to the secondary metabolic cucurbitacin content.[4]

Present investigation is about Phytosterol which is widespread in plants have structural similarity to cholesterol.[5,6] Qualitative analysis of Phytosterols with chemotaxonomic comparison and antioxidant activity gives more assessment to the selected species of family Cucurbitaceae.

MATERIALS AND METHODS

Plant Material

Fresh fruits of *Momordica charantia* L., *Trichosanthes dioica* Roxb., *Coccinia grandis* (L.) Voigt, *Lagenaria siceraria* (Molina) Standl., *Cucirbita maxima* Duchesne were collected from market were authenticated from department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. The fruits were washed well, using tap water and distilled water twice and dried in shade for a period of 20 days, at an ambient temperature of 35˚C. After drying the fruits, the seeds were separated by cutting them into small pieces to avoid its

intercession. The dried seeds were ground properly using a motor and pestle and later using a grinder, to obtain the powdered and fibrous form.

Preparation of Plant Extract

Powdered seeds were made to undergo Soxhlet's extraction (12g in 250ml of solvent) in solvent Chloroform. (The extracts were named as CM, MC, TD, CG and LS).

Qualitative Phytochemical Screening

Extracts were analyzed for the presence of phytosterol by Solkowski test and Liebermann Burchard's test.

Solkowski Test[7]

2 ml extract taken in a test tube. 2ml Chloroform and 2ml conc. Sulphuric acid was added in it. Brown or red colored ring on the Sulphuric acid layer given the confirmatory test for Phytosterols.

Liebermann Burchard's Test[8]

2ml extract was taken in a test tube. Chloroform (2ml) Acetic Anhydride (2ml) and conc. Sulphuric acid (2ml) was added in it. Translucent green colored tint given the confirmatory test.

TLC (Thin Layer Chromatography) Chromatography

The adsorbent used for preparation of thin layer plate as a stationary phase was Silica Gel G. 15 g powder of Silica Gel G was mixed with 30ml Distilled water. This Silica Gel G suspension was spread with a spreader on thin layer chromatographic glass plates fixed on a stage. The prepared plates were air-dried and activated in an oven at 110^{o}C for 30 min. The activated plates then used for the application of samples and standard β-sitosterol (Standard-MP Biomedicals) solutions. Aliquots of each of the extracts were separately applied (samples and standard) to the plate as a 6 mm wide band, 8 mm from the bottom. The 10 ml mobile phase consisted of Benzene : Ethyl Acetate (5:1). Development was carried out in a twin glass chamber saturated with the mobile phase and derivatization was carried out with Anisaldehyde : Sulphuric Acid : Galacial Acetic Acid (0.1:0.2:0.3) and after which the plate was heated at 110°C for 10 min and viewed under normal light.

The Rf values of Standard spot and Sample spots were calculated by the formula:

$$\textbf{Rf Value} = \frac{Distance\ travelled\ by\ the\ Compound}{Distance\ travelled\ by\ the\ Solvent}$$

DPPH Scavenging Activity

The free radical scavenging activity of the extracts was measured in vitro by 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) assay. A 0.36 mg/ml stock solution of the same was prepared with methanol and stored in the dark at 20°C. 3 ml of this solution was mixed with 100 µl of each of the plant extracts (100 µg/ml). Three concentrations 100, 200, 300 µg of dried Chloroform plant extract and standard Ascorbic Acid were prepared in Methanol. 500µl of freshly prepared 0.1mM DPPH solution (in methanol) was added in each extract/standard containing test tube. The change in color from purple to yellow was measured spectrophotometrically at 517nm after 30 mins. Initial reading of DPPH as positive control and Methanol as negative control was noted before.

The scavenging of DPPH radical was calculated with following formula:

DPPH Radical Scavenging Activity (%)= $\frac{Ac-As}{Ac} \times 100$

Where,

Ac= The Absorbance in control (DPPH radical +Methanol)

As= The Absorbance in sample (DPPH radical+Sample/Standard)

Chemotaxonomy

The retention factor (Rf) values for the detected spots were determined from TLC and the Paired affinity indices (PAI), Grouped Affinity (GA) and Isolation Value (IV) were calculated according to Ellison *et al.* (1962) as follows[9]:

1. PAI = $\frac{\text{Spots common in species A and B}}{\text{Total spots in A and B}} \times 100$

2. GA = Total PA Value + 100

3. IV = $\frac{\text{Number of unique spot in a species}}{\text{Total number of spots in all species}} \times 100$

RESULTS AND DISCUSSIONS

Preliminary Phytochemical Test for Phytosterols

According to the reports of Tiwari, *et al.* (2011) and Venkata, *et al.* (2010), Salkowski's Test and Liebermann Burchard's Test are the two important test for the detection of sterols from the plant material.[10,11]

Salkowski and Liebarmann Burchard Tests were done for the confirmation of Phytosterols in the selected plants. Salkowski test had given Brown to red colored ring on the sulphuric acid layer while Lieberman Burchard Test given the translucent Green colored tint, which confirmed the presence of phytosterols in all the selected plant materials CM, LS, TD, CG and MC (Table 1).

Table 1: Preliminary Phytochemical Test.

Plant Material	Solkowski test	Liebermann Burchard's test
Momordica charantia (MC)	+	+
Coccinia grandis (CG)	+	+
Cucurbita maxima (CM)	+	+
Tricosanthes dioica (TD)	+	+
Lagenaria siceraria (LS)	+	+

Thin Layer Chromatography (TLC)

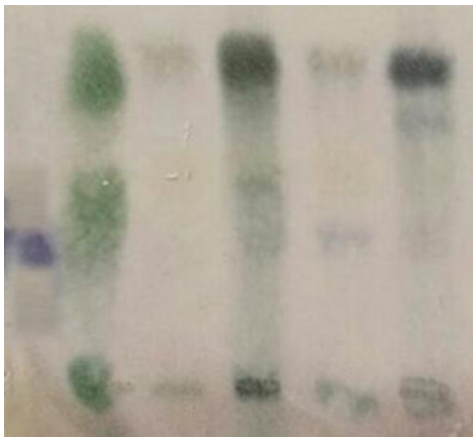
TLC is used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound. Additional tests involve the spraying of phytochemical screening reagents, which cause colour changes according to the phytochemicals existing in a plants extract; or by viewing the plate under the UV light.[12]

In the present research work, detection of phytosterol was validated by TLC band with Rf value 0.29 of standard β-Sitosterol and seed extracts CM, LS, TD, CG and MC (Table 2, Plate 1).

Table 2: Rf Values.

Track No.	RF Values
Standard (β-Sitosterol) (S)	**0.29**
Momordica charantia (MC)	**0.29**, 0.41, 0.52, 0.58
Coccinia grandis (CG)	**0.29**, 0.41, 0.58
Cucurbita maxima (CM)	0.05, **0.29**, 0.41, 0.58, 0.70
Tricosanthes dioica (TD)	**0.29**, 0.41, 0.58, 0.58, 0.64
Lagenaria siceraria (LS)	**0.29**, 0.41, 0.64

Plate 1: Thin Layer Chromatography.



Track No.	Track Name
S	Standard (β-Sitosterol)
1	*Momordica charantia*(MC)
2	*Coccinia grandis* (CG)
3	*Cucurbita maxima* (CM)
4	*Tricosanthes santhes* (TD)
5	*Lagenaria siceraria* (LS)

Antioxidant Activity

The absorbance obtained in all selected seed extract *Tricosanthes dioica, Cucurbita maxima, Coccinia grandis, Momordica charantia and Lagenaria siceraria* and Standard (AA) in different doses (100µg/ml, 200µg/ml, 300µg/ml) was found to be less than that of the absorbance in the control (DPPH) (Table 3). As concentration increase, %Scavenging found to be increasing. The highest %Scavenging among all selected plant materials was found to be in *Tricosanthes dioica* which was **43.96%** in 200µg/ml *and* lowest % Scavenging was observed in *Momordica charantia* which was **17.55%** in 100µg/ml (Table 3 and Figure 1).

Table 3: Percent Scavenging Avtivity. Each value represents Mean ± SEM (n=3).

Sr. No	Name of sample	Doses µg/ml	Percent Scavenging Avtivity
1	Ascorbic acid	100	90.90 ± 0.038
		200	92.87 ± 0.02
		300	93.95 ± 0.01
2	*Tricosanthes dioica*	100	42.48 ± 0.03
		200	43.97 ± 0.01
		300	47.47 ± 0.06
3	*Cucurbita maxima*	100	25.28 ± 0.01
		200	40.20 ± 0.03
		300	41.86 ± 0.17
4	*Coccina grandis*	100	23.98 ± 1.98
		200	34.29 ± 0
		300	37.66 ± 0.053
5	*Momordica charantia*	100	17.33 ± 0.20
		200	19.17 ± 0.10
		300	22.63 ± 0.01
6	*Lagenaria siceraria*	100	25.14 ± 0.02
		200	26.05 ± 0.03
		300	26.81 ± 0.05

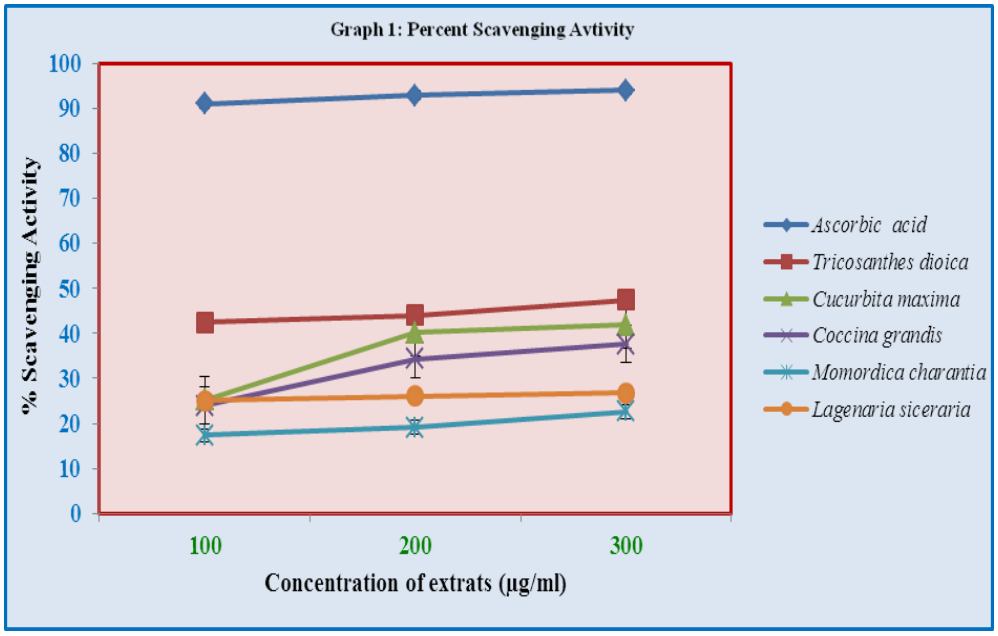


Figure 1: DPPH radical scavenging assay data represents, mean ±SEM, for n=3.

Chemotaxonomic Calculation

Table 4: Paired Afinity Index.

Track Name	*Cucurbita maxima*	*Lagenaria siceraria*	*Tricosanthes dioica*	*Coccinia grandis*	*Momordica charantia*	Total PA
Cucurbita maxima	100	125	33.3	37.5	30	225.8
Lagenaria siceraria		100	42.8	33.3	25	201.1
Tricosanthes dioica			100	42.8	33.3	176.1
Coccinia grandis				100	42.8	142.8
Momordica charantia					100	100

In order to assess the taxonomic significance of the previously mentioned separated compounds in the systematic of the studied species with reference to the angiosperm phylogeny group (APG III, 2009), different taxonomic levels were investigated. Comparison of the paired affinity indices between the different genera indicated that some species are closely related.

Paired Affinity Indices (PAI) is the ratio expressed in percentage of spots common in species A and B to the total number of spots in A and B (A and B are two individual species to be onsider) (From TLC Rf values-Table 2). The total number of spots obtained in all the species was 20. The PA value calculated on the basis of presence and absence of the phytosterols as shown in Table 4. The highest PA value (42.8%) was observed between *Lagenaria siceraria* and *Tricosanthes dioica, Tricolanaria dioica and Coccinia grandis, Coccinia grandis and Momordica charantia* which shows that these genera are closely related. The lowest PA value

(25%) was observed between *Cucurbita maxima and Lagenaria siceraria, Lagenaria siceraria and Momordica charantia*.

The number of Steroid spots varied from 4 to 5. The lowest number of spots were found in *Coccinia grandis* and *Lagenaria siceraria* and the highest number of spots were in *Cucurbita maxima* and *Momordica charantia* (Table 2).

The geometrical shapes of the polygonal graphs of the studied taxa, reflects the same pattern shown by the phytosterol constituents in the selected seed extarctions (Figure 2). Figure 2 shows the polygonal representation of the phytosterol constituents within the 5 selected taxa of family Cucurbitaceae.

Group Affinity (GA) is the Total Paired Affinity Index (PAI) of all the species. GA value was also showed the close relationship between *Lagenaria siceraria* and *Tricosanthes dioica*. Isolation Value (IV) is the ratio expressed in percentage of number of unique spots in a species to total number of spots in all species (Table 5).

Table 5: GA and IV Value.

Plant material	GA Value	IV Value
Cucurbita maxima	205	0.1
Lagenaria siceraria	201.23	0
Tricosanthes dioica	201.16	0
Coccinia grandis	200.58	0
Momordica charantia	100	0.05

CONCLUSION

The simple technique of Thin Layer Chromatography (TLC) has been used in the study of different species and chemical interrelation between them. This research work has revealed Phytosterol potential of seeds extract of Cucurbitaceae family of using TLC and PA value. The highest PA value was seen between *Lagenaria siceraria* and *Tricosanthes dioica* which is indication of close chemical affinity between them. Higher PA value can be considered as a marker of close relationship. Also the paper divulge the antioxidant activity of the Cucurbitaceae seeds.

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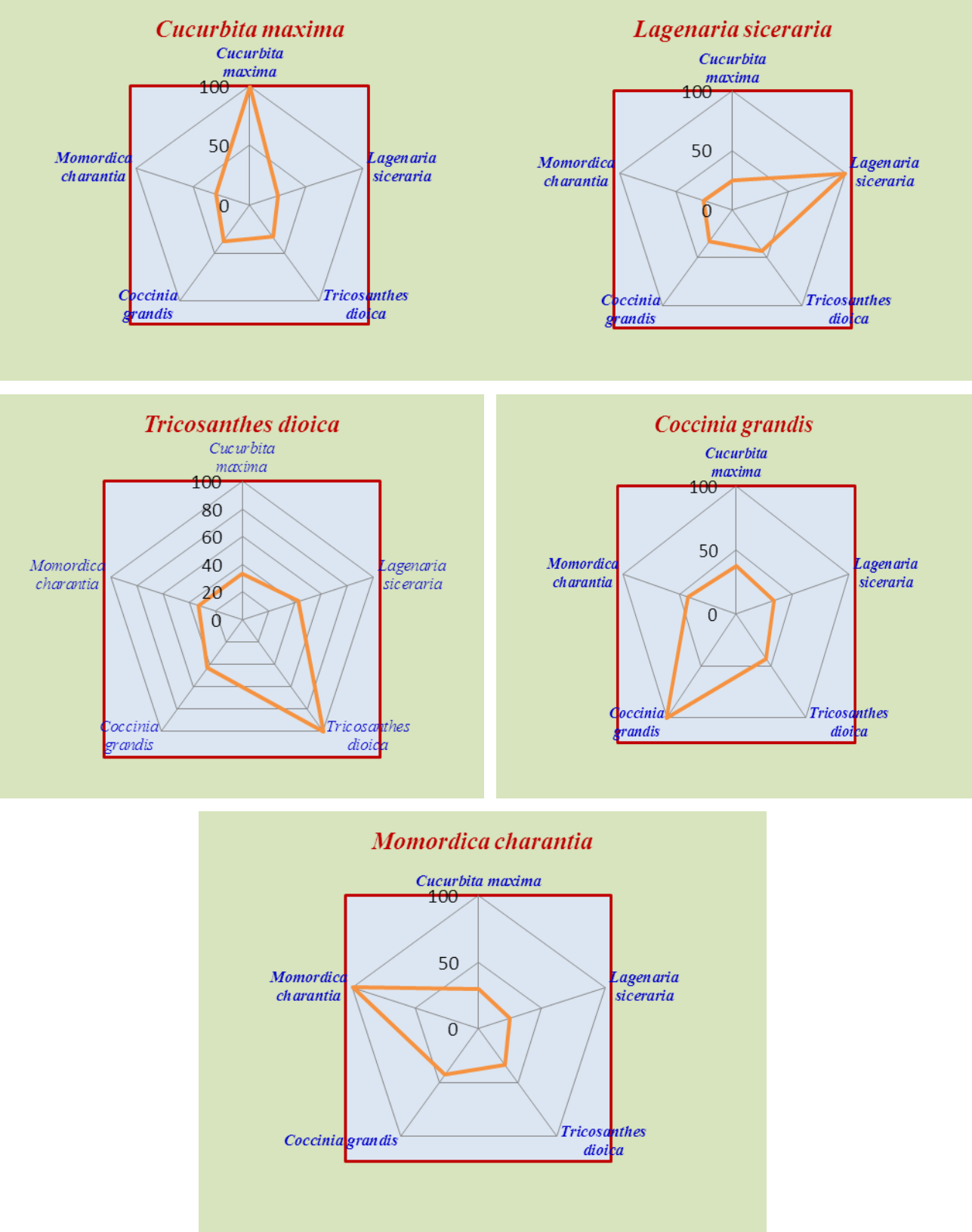

Figure 2: PAI of selected seeds.

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