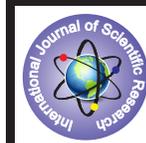


Phytochemical Affinities of Triterpenoids of Some Edible Seeds and Identification of Phytosterols as a Nutritional Attribute.



Botany

KEYWORDS : Triterpenoid, Paired Affinity Index, Phytosterols, β -sitosterol, Nutrients.

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ABSTRACT

The seed extracts of *Punica granatum L.* (Pomegranate), *Citrullus lanatus var. lanatus* (Watermelon), *Linum usitatissimum L.* (Flax), *Glycine max (L.) Merr.* (Soybean), *Helianthus annuus L.* (Sunflower), *Trigonella foenum-graecum L.* (Fenugreek), *Sesamum indicum L.* (Sesame), *Cuminum cyminum L.* (Cumin), *Trichosanthes dioica Roxb.* (Parwal), *Citrullus fistulosus Stocks* (Dhense), and *Cucurbita maxima Duchesne* (Pumpkin) were evaluated for identification and separation of different triterpenoid compounds especially phytosterols using Thin Layer chromatography and Paired Affinity Index methods. The Solkowaski test was performed to identify the triterpenoid while Paired Affinity Index was used to calculate and correlate the chemical affinities between all the selected taxa. Phytosterols (a class of triterpenoid) play an important role in cell membrane function of plant and biosynthesized via mevalonate pathway. Phytosterols profusely found in seeds and fruits while meager quantity observed in other part of plant. In study of TLC Benzene: ethyl acetate (5:1) solvent system showed violet blue colored bands (Rf 0.59 to 0.60) 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. Edible seeds containing plant sterols adds a new dimension in nutritive value apart from nutrients like carbohydrates, proteins, oils, minerals like calcium, potassium, etc., and can be a important diet supplements as nutrients of food.

1. Introduction:

The traditional medicines involve the use of different plant extracts and the bioactive constituents secondary metabolites are responsible for medicinal activity of plants [1]. Biosynthesis of secondary metabolites from intact plants, plant parts, tissue cultures has gained increasing attention over the years.

Plant derived substances have become of great interest owing to their versatile applications. Triterpenes are a large class of compounds that include steroids and sterols. This class is present abundantly in plants and animals. They have a C30 carbon skeleton and most naturally occurring triterpenoids are biosynthesized from squalene [2]. Breast cancer is hormone signaling failures-related disease and the second leading cause of female cancer mortality. Among plant-based agents, triterpenoids have emerged as a promising group of phytochemicals with proposed anti-cancer activity. They are the major components of some oriental and traditional medicine herbs widely distributed all over the world. Triterpenoids are relatively non-toxic and could be used as chemopreventive / chemoprotective agents in clinical praxis. Numerous studies using cell culture assays and animal models of cancer have revealed that triterpenoid holds great potential not only in the therapy of breast cancer but also in preventing this disease [3]. Phytosterols and phytosterols are a large group of compounds that are found exclusively in plants [1]. They are a group of steroid alcohols and esters that occur naturally in plants. Phytosterols can be found at widely varying concentrations in the fat-soluble fractions of roots, stems, branches, leaves, seeds and blossoms etc. They are constituents of both edible and ornamental plants, including herbs, shrubs and trees [4]. Phytosterols inhibit the intestinal absorption of cholesterol [5]. The use of foods containing phytosterols is a relatively recent development in human nutrition³. Phytosterols, as functional ingredients in foods, reduce total cholesterol and LDL-cholesterol levels through a reduction in cholesterol absorption [6]. Plant sterols known as phytosterols have the same function in plants like cholesterol have in an animal, that is to play an important role in cell membrane function [7-9].

1.1. Biosynthesis of Phytosterols:

Plant triterpenoids, comprise primary metabolites, such as the Phytosterols and the brassinosteroid and the secondary metabolites, such as saponins. The isopentenyl pyrophosphate (IPP) is generated by triterpenoid biosynthesis through the cytosol,

peroxisome and endoplasmic reticulum-localized MVA pathway. **Squalene** is the further intermediate which is ultimately form into phytosterols. In higher plants, the biosynthesis begins with the generation of isopentenyl pyrophosphate (IPP), the principal precursor, through the mevalonate (MVA)/3-hydroxy-3-methylglutaryl- CoA reductase (HMGR) pathway or the 2-C-methyl-Derythritol 4-phosphate (MEP)/1-deoxy-D xylulose 5-phosphate (DOXP)/non-MVA pathway. The IPP is isomerized to its allylic isomer dimethylallyl pyrophosphate (DMAPP). The consecutive condensation of IPP and DMAPP units leads to the formation of prenylated pyrophosphates, the immediate precursors of the different terpenoid classes (Fig. 1). These condensation reactions are catalyzed by specific prenyltransferases which are named according to the product they generate. Specific terpenoid synthases then modify these precursors to terpenoid skeletons [10], which are subsequently decorated by various enzymatic modifications to generate the structural and functional diversity of terpenoids. Plants also exhibit a clear compartmentalization for the generation of IPP and the synthesis of terpenoids (Fig. 1) [11-12]. The 'head-to-tail' condensation of two IPP units with a DMAPP unit yields the C15 farnesyl pyrophosphate (FPP), two of which subsequently fuse 'head-to-head' to generate the linear C30 triterpenoid precursor, squalene. This compound is further epoxidized to 2, 3-oxidosqualene (Augustin et al., 2011), which, in turn, is typically cyclized by specific oxidosqualene cyclases (OSCs) to tetra- or pentacyclic structures to form the damarenes, tirucallanes and phytosterols.

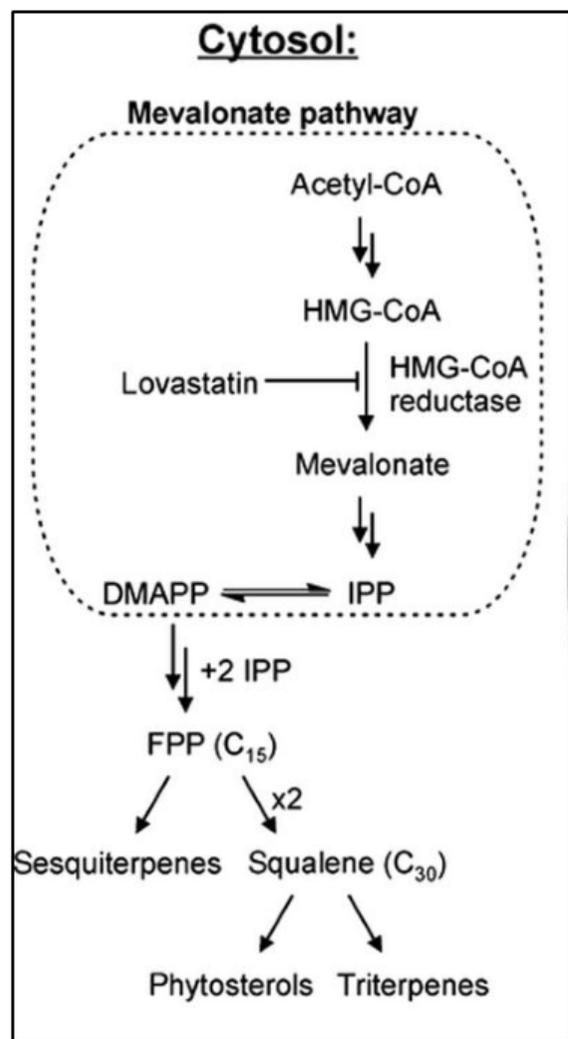


Fig.1: Malvonate Pathway: Biosynthesis of Phytosterols.

The most commonly found phytosterols is being β -sitosterol, stigmasterol and campesterol [13]. Phytosterols are bioactive components occurring in all vegetable foods, and make up the greatest proportion of the non-saponifiable fraction of lipids [14]. Chemical structure of these sterols is similar to cholesterol. Plant oils are particularly rich source of phytosterols, however all sources are thought to be effective in the treatment or prevention of high cholesterol or hypercholesterolemia.

β -sitosterols is the major phytosterols synthesized mostly in higher plants and animals obtain them through diet [15]. A proper diet is essential from the very early stages of life for proper growth, development and to remain active. Food consumption, which largely depends on production and distribution, determines the health and nutritional status of the population. Nutrition is a basic human need and a prerequisite to a healthy life.

The body needs some essential nutrients in relatively large amounts. These macronutrients include protein, fat, carbohydrate, and water. Micronutrients, such as vitamins and minerals, are required in much smaller amounts. Presence of β -sitosterol in soybean has been reported to suppress carcinogenesis. It can also be the factor used to form the lympho cells and NK in the immunity process circulation [16-17].

Seeds are the embryo and food supply of new plants, whereas nuts are dried tree fruits, which are contained within hard

shells. Both seeds and nuts contain 10-25 percent protein and they are high in mono and polyunsaturated fat. They are good sources of fiber, vitamins; thiamine (B1), riboflavin (B2) and E and the minerals; calcium, phosphorus, potassium and iron. (Legume seeds). Many seeds are edible and the majority of human calories come from seeds, especially from legumes and nuts. Many epidemiological studies of groups consuming diets rich in vegetables and fruits have indicated a reduced incidence of various types of cancer, cardiovascular disease, diabetes, and other chronic diseases [18].

1.2. Nutraceutical value of selected seeds:

Trichosanthes dioica Roxb. (Parwal) (Family - Cucurbitaceae) is used as tonic, febrifuge, in edema, alopecia, and in subacute cases of enlargement of liver. Two main phytosterols present in *T. dioica* are, namely, 24 α -ethylcholest-7-enol and 24 β -ethylcholest-7-enol. Seeds of *T. dioica* also contain lectin, a carbohydrate (specifically galactose) binding protein [19].

Punica granatum L. (Pomegranate) (Family - Lythraceae) is a rich source of antioxidants. Therefore, it helps to protect your body's cells from free radicals, which cause premature aging [20].

Cuminum cyminum L. (Cumin) (Family - Apiaceae) seeds possess numerous phyto-chemicals that are known to have antioxidant, carminative and anti-flatulent properties. It also contains very good amounts of B-complex vitamins such as thiamin, vitamin B-6, niacin, riboflavin, and other vital anti-oxidant vitamins like vitamin E, vitamin A and vitamin C. The seeds are also rich source of many flavonoid phenolic anti-oxidants such as carotenes, zeaxanthin, and lutein [21].

Sesamum indicum L. (Sesame) (Family - Pedaliaceae) seeds are rich source of protein and also one of the first crops processed for oil production. Sesame seeds also contain two unique substances: sesamin and sesamol known to have a cholesterol lowering effect in humans and to prevent high blood pressure.

The nutritive value of the immature pods of *Glycine max* (L.) Merr. (Soybean) (Family - Fabaceae) are rich in vitamins (B1, B2), minerals (iron, calcium, phosphorus), and protein content [22].

Linum usitatissimum L. (Flax) (Family - Linaceae) is rich in w-3 fatty acid and also in fiber and protein, high content of a-linolenic acid, dietary fiber mucilage, lignans and phenolic compounds, all of which are probably beneficial in reducing the risk factors for both coronary heart diseases and cancer [23].

Every aspect of the fruit *Citrullus lanatus* var. *lanatus* (Watermelon) (Family - Cucurbitaceae) has nutritional value, including the rind and the seeds. The dried egusi seed without include: water, protein, fats, carbohydrate, Calcium, Phosphorous, iron, thiamin, riboflavin, niacin, folate. The seed being an excellent source of energy.

The fruit *Cucurbita maxima* Duchesne (Pumpkin) (Family - Cucurbitaceae) is a good source of β -carotene and has moderate content of carbohydrates, vitamins and minerals [24]. Consumption of foods containing carotene helps in prevention of skin diseases, eye disorders and cancer [25]. The pumpkin seeds are rich sources of lipids, fibre and proteins.

Seeds of *Helianthus annuus* L. (Sunflower) (Family - Asteraceae) are an important source of oil, protein, calcium, carbohydrate and ash [26].

Seeds and green leaves of *Trigonella foenum-graecum* L. (Fenu-greek) (Family - Fabaceae) are used in food as well as in medicinal application which is an old practice of human history. It provides natural food fibre and other nutrients required in human

body [27].

The fruit *Citrullus fistulosus* Stocks (Dhense) (Family - Cucurbitaceae) contain proteins, fats, carbohydrates, fibre, moisture in a moderate amount [28].

1.3. Chemotaxonomy:

The classification of plants results in an organized system for the naming cataloging of future specimens, and ideally reflects scientific idea about interrelationships between plants is known as taxonomy [29]. The approach of taxonomy in which chemical nature of plants is used in developing classification or solving taxonomical problems is called chemotaxonomy. It gives close relationship between chemical constituents of plants and their taxonomical status [30]. When a moderately large group of species is being compared, "Paired Affinity (P.A.) Indices" can be a good tool. In this species were compared on the basis of their chemical affinity. The method adopted by Ellison et al. was followed to make suitable comparison in the form of qualitative relationships [31].

Present investigation is the testing of those seeds of edible plants which contain phytosterols. Solkowski test was done for the preliminary estimation of phytosterols and presence of phytosterols was confirmed by comparing sample with standard β -sitosterol by Thin Layer Chromatography. The work also describes ways of presenting data concerning the occurrence of chemical constituent triterpenoids of various taxa of some genus and related genus in order to provide indices of potential value in ascertaining taxonomic affinities.

2. Materials and Methods:

2.1. Plant material and Extraction:

The plants and seeds of *Punica granatum* L. (PS1), *Citrullus lanatus* var *lanatus* (PS2), *Linum usitatissimum* L. (PS3), *Glycine max* (L.) Merr. (PS4), *Helianthus annuus* L. (PS5), *Trigonella foenum-graecum* L. (PS6), *Sesamum indicum* L. (PS7), *Cuminum cyminum* L. (PS8), *Trichosanthes dioica* Roxb. (PS9), *Citrullus fistulosus* Stocks (PS10), *Cucurbita maxima* Duchesne (PS11) were collected and authenticated in the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur and was studied from Nagpur flora [32]. Seeds were separated from plant washed, spread, and shed dried on concrete floor. Dried seeds weighing 100g from plants were grind to form coarse powder. The powdered materials were extract using cold maceration technique by suspending 100g of powder material in 500 mL of Hexane in wide mouth glass bottle kept for 24 hr. Obtained extracts were concentrated under reduced pressure dried and stored in glass bottle at 25°C and labeled respectively PS1 to PS11. Extracts PS1 to PS11 were tested for the presence of phytosterols and showed positive Solkowski test [33].

2.2. Thin Layer chromatography:

Chromatographic analysis can be used to identify the important compounds from plant. Thin layer chromatography is the method used to separate various compounds present in the plant.

Extracts showed positive test were studied using thin layer chromatography comparing with standard β -sitosterol (Standard-MP Biomedicals) for confirmation of phytosterols. TLC plates (TLC Silica gel 60 F₂₅₄, 20x20 Merk) were used. The Benzene : Ehtyl Acetate (5:1) was finalized among different solvent system tried for sharp bands and accurate Rf. TLC plates were activated with methanol and dried. Extracts PS1 to PS11 and standard β -sitosterol about 1mg were dissolved in 5mL of Hexane in a test tube respectively. Spots were applied 1.5 cm above from bottom 0.5 cm from side and neighboring spot on plate. Plates were placed in a glass chamber which was saturated with solvent Benzene : Ehtyl Acetate (5:1). TLC plate were removed from chamber, air dried and sprayed by Anisaldehyde : Sulphuric

Acid : Galcial Acetic Acid (0.1:0.2:10) reagent and dried at 100°C in oven for 10 minutes.

2.3. Paired Affinity Index (PAI):

Paired Affinity Indices (PAI) is a measure of chemical affinity between two taxa [34-36]. PAI between A and B, Group affinity (GA) and Isolation Value (IV) were calculated as follows:

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

$$GA = \text{Total PA Value} + 100$$

$$IV = \frac{\text{Number of Unique spots in a species}}{\text{Total number spots in all species}} \times 100$$

3. Results and Discussion:

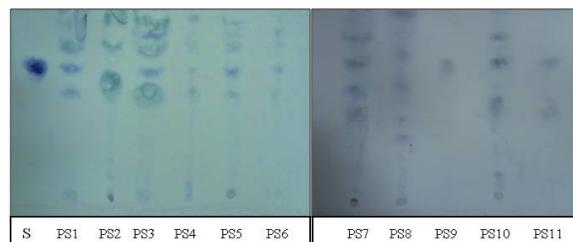


Fig 2: Thin Layer Chromatography Fingerprints of Extracts (PS1 to PS11) and standard β -sitosterol (S)

In the present investigation a number of triterpenoid compounds were spotted but they were not identified qualitatively. Chromatographic spots are regarded as excellent markers and are much more important than the chromosome numbers in taxonomy of plants (Grant 1968).

11 seeds showed positive preliminary Solkowski test for triterpenoids. Violet blue colored bands of sterols were appeared on TLC plate.

Thin layer chromatography showed the maximum separation in solvent system. Total number of spots obtained in all plant species was 34 out of which 1spot was found in PS1, 2 were found in PS11, 5 were found in PS1,PS2, PS3, PS6 and PS10, 6 were found in PS4, PS5, PS7 and PS8 with Rf values given in Table 1. PAI is the ratio expressed in percentage of spots common in any two species to the total spots in those two species. The PA value calculated on the basis of presence and absence of different compounds (Table 2).

The highest PA value was found to be 36.36% observed between PS4 - *Glycine max* (L.) Merr. (Soybean) & PS3 - *Linum usitatissimum* L. (Flax) which showed that they are chemically closely related. The lowest PA value was 8.33% observed between PS5 - *Helianthus annuus* L. (Sunflower) & PS4 - *Glycine max* (L.) Merr. (Soybean), PS8 - *Cuminum cyminum* L. (Cumin) & PS7 - *Sesamum indicum* L. (Sesame) and PS8 - *Cuminum cyminum* L. (Cumin) & PS4 - *Glycine max* (L.) Merr. (Soybean) which showed that they had lowest chemical relativity. *Trichosanthes dioica* Roxb. (PS9) and *Cucurbita maxima* Duchesne (PS11) plants belong to the same family Cucurbitaceae and it was seen that there is very less difference in the Rf values found in these plants, which can be considered as same. So, can be considered as a close chemical affinity with each other for that particular compound with Rf value 0.65, 0.66 respectively. But individually these plants did not show any affinity with other plants. Thus, reasonable chemical affinities were noted between the species. It is found that the chemical similarities between certain species known to be morphologically related.

The number of spots varied from 1 to 6 in the eleven plants was studied. The lowest number of spots was found in *Trichosanthes*

dioica Roxb. (Parwal) i.e. 1 while highest number of spots i.e. 6 was found in 4 plants- *Glycine max* (L.) Merr. (Soybean), *Helianthus annuus* L. (Sunflower), *Sesamum indicum* L. (Sesame), *Cuminum cyminum* L. (Cumin). PAI can be best illustrated in the form of polygonal graph (Fig 3).

Group Affinity (GA) is the Total Paired Affinity Index (PAI) of all the species. GA value was also showed the close relationship between PS4 - *Glycine max* (L.) Merr. (Soybean) & PS3 - *Linum usitatissimum* L. (Flax). 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 1: Rf Values of all spots present in all plant samples.

Track No.	Rf values
PS1	0.49, 0.6, 0.69, 0.81, 0.86
PS2	0.12, 0.2, 0.54, 0.71, 0.81
PS3	0.5, 0.59, 0.68, 0.74, 0.8
PS4	0.08, 0.49, 0.59, 0.68, 0.74, 0.8
PS5	0.46, 0.56, 0.68, 0.73, 0.78, 0.85
PS6	0.46, 0.59, 0.69, 0.79, 0.85
PS7	0.42, 0.54, 0.62, 0.79, 0.82, 0.86
PS8	0.17, 0.32, 0.57, 0.74, 0.83, 0.92
PS9	0.65
PS10	0.1, 0.22, 0.49, 0.62, 0.79
PS11	0.43, 0.66

Table 2: Paired Affinity Indices (PAI) of different species

Track No.	PS1	PS2	PS3	PS4	PS5	PS6	PS7	PS8	PS9	PS10	PS11
PS1	100	10	0	9.091	0	10	0	0	0	10	0
PS2		100	0	0	0	0	9.091	0	0	0	0
PS3			100	36.36	9.091	10	0	9.091	0	0	0
PS4				100	8.33	10	0	8.33	0	9.091	0
PS5					100	18.18	0	0	0	0	0
PS6						100	9.091	0	0	9.091	0
PS7							100	8.33	0	18.18	0
PS8								100	0	0	0
PS9									100	0	0
PS10										100	0
PS11											100

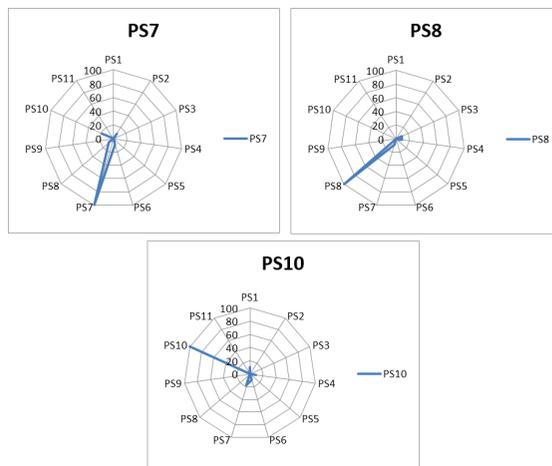
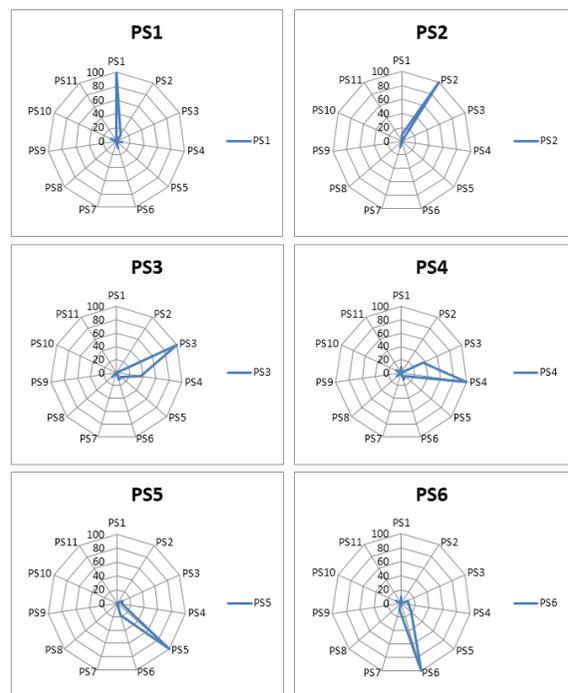


Fig 3: PS1- PS8 and PS10: Polygonal Representation of the Paired Affinity Indices of Each texa to all other. Affinity indices are expressed along the radii from 0% to 100%, beginning at the centre. Polygon for PS9 and PS11 is not expressed as they showed zero affinity with any other texa.

Band of standard β -sitosterol appeared to be of violet blue colored (Fig 2). Thus all the violet blue colored bands in all samples which appeared in the same row of violet blue colored β - sitosterol represent the presence of phytosterols.

One of the band in each seed sample track having Rf value nearly same as that of the Rf value of the standard (Table 4). This can conclude that the seed samples taken contain β -sitosterol.

Table 3: Group Affinity (GA) and Isolation Value (IV) of triterpenoid compounds.

Plant Sample	GA Value	IV Value
PS1	139.091	5.9
PS2	119.091	8.9
PS3	173.633	2.9
PS4	181.202	2.9
PS5	135.601	8.9
PS6	166.362	0
PS7	144.692	5.9
PS8	125.751	11.8
PS9	100	2.9
PS10	146.362	5.9
PS11	100	5.9

Table 4: Rf values of Thin layer Chromatography for Phytosterols

Track No.	Name of plant (seeds)	Rf value
S	<i>β-sitosterol</i>	0.6
PS1	<i>Punica granatum</i> (Pomegranate)	0.6
PS2	<i>Citrullus lanatus</i> (Watermelon)	0.54
PS3	<i>Linum usitatissimum</i> (Flax)	0.59
PS4	<i>Glycine max</i> (Soybean)	0.59
PS5	<i>Helianthus annuus</i> (Sunflower)	0.56
PS6	<i>Trigonella foenum-graecum</i> (Fenugreek)	0.59
PS7	<i>Sesamum indicum</i> (Sesame)	0.54
PS8	<i>Cuminum cyminum</i> (Cumin)	0.57
PS9	<i>Trichosanthes dioica</i> (Parwal)	0.65
PS10	<i>Citrullus fistulosus</i> (Dhense)	0.62
PS11	<i>Cucurbita maxima</i> (Pumpkin)	0.66

Various seeds which are usually important dietary food for people, contain the nutrients like protein, fat, carbohydrate, water, vitamins, minerals, etc. act as dietary supplements. Studies on

such seeds until, includes the knowledge only about nutritive compounds like Carbohydrates, Fats, Proteins, Minerals, Vitamins, etc. This research thus gives a new vision about the addition of phytosterols as the major nutrients of food of the people with various advantages of it (phytosterols) (Fig3).

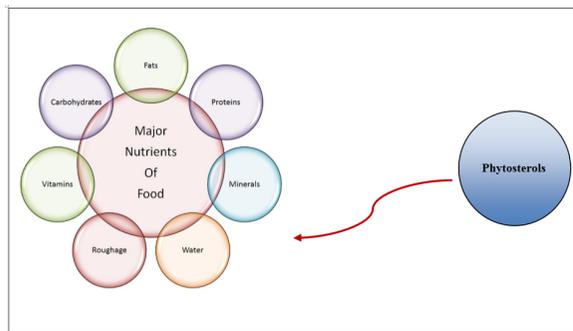


Fig 3: Phytosterols as a Nutritional Attribute

4. Conclusion:

The simple technique of Thin Layer Chromatography (TLC) has been used in the study of different species and chemical inter-relationship between them. This research work has revealed triterpenoid potential of some seeds of by using TLC and PA value. The highest PA value was seen between *Glycine max* (L.) Merr. (Soybean) & *Linum usitatissimum* L. (Flax) 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 that phytosterols can be added as one of the nutritional attribute with other important nutrients like carbohydrates, proteins, fats, minerals, vitamins, roughage, water, etc. in the different edible foods.

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