The Story of Beta-sitosterol - A review

ABSTRACT

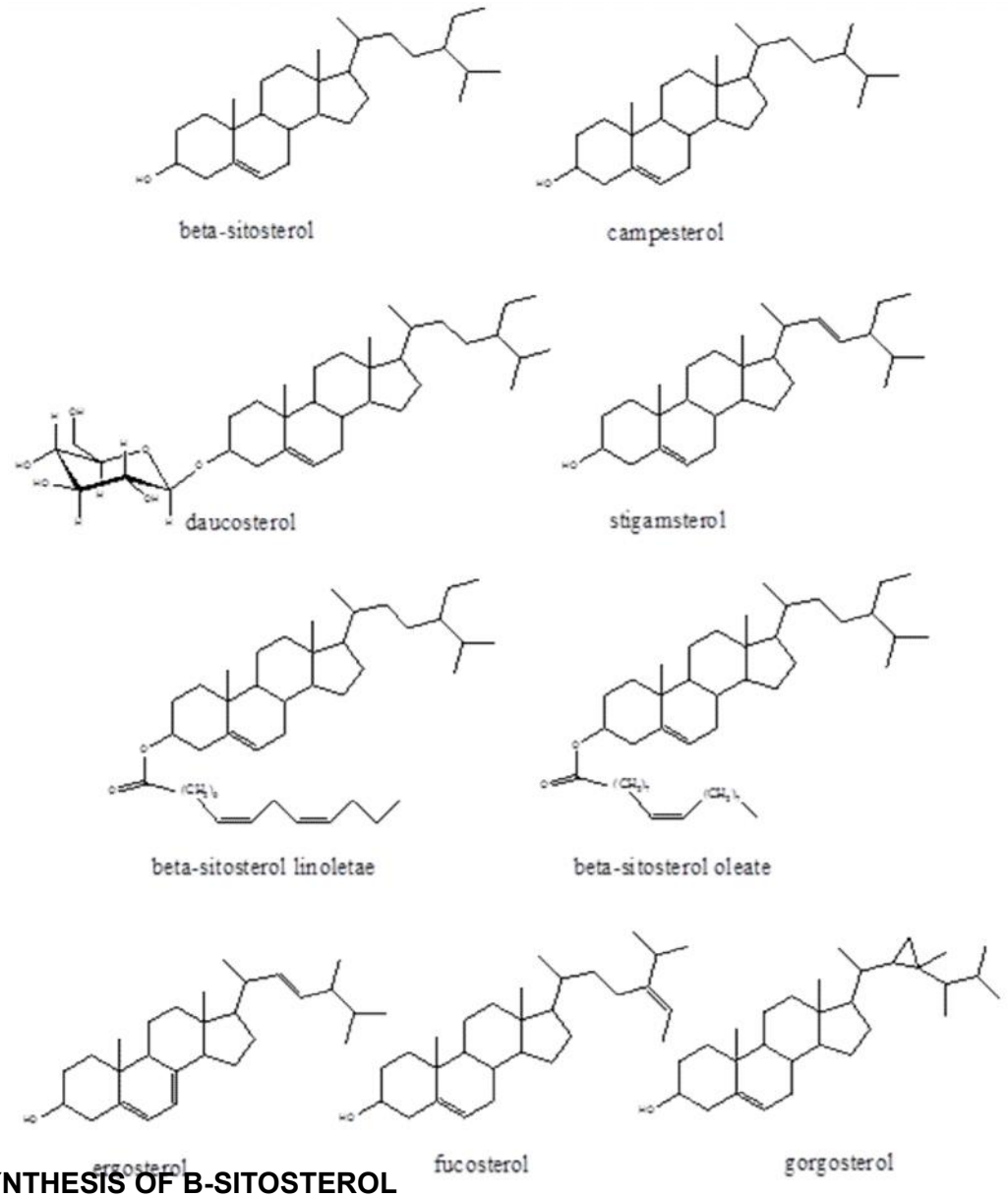
Aims: Phytosterols are a subgroup of the steroids, as an important class of bioorganic molecules, widespread in plants, animals, marines as well as fungi, and have similarity to cholesterol in structure. These compounds have a long history of consumption as food or pharmaceutical products, and generally recognized as safe without undesirable side effects.
Place and Duration of Study: Medicinal plants Research Center and Pharmaceutical Sciences Research Center, between March 2013 and May 2013.
Results: Among them, β-sitosterol is usually used for heart disease, hypercholesterolemia, modulating the immune system, preventing of cancer, as well as for rheumatoid arthritis, tuberculosis, cervical cancer, hair loss and benign prostatic hyperplasia. Furthermore, diverse biological activities are observed using these natural compounds or the extracts, in which implicated, e.g. antimicrobial, trypanocidal, mosquito larvicidal even as neutralizing agent on viper and cobra venom.
Conclusion: Some of the above indications are evidence based, but others are still in doubt and needed more investigations to confirm its efficacy and safety. Regarding to the importance of these natural sterols and β-sitosterol as the most abundant of them, the main pharmacological and biological activities together with their clinical trials is reviewed here.

Keywords: β-sitosterol; Phytosterols; Pharmacological acyivitiea; Biological activities.

1. INTRODUCTION

A term “Phytochemicals'” (plant based chemicals), was introduced to the world in1994 and promptly became a trend and frontier for researchers and scientists, of which phytosterols are a subgroup of the steroids as an important class of bioorganic molecules. Phytosterols are widespread in plants and animals as well as fungi, and have structural similarity to cholesterol. Phytosterols play essential roles in the physiology of eukaryotic organisms. For instance, cholesterol is the main part of the cellular membrane in animals, affecting the cell membrane's fluidity and serving as secondary messenger in developmental signaling [1]. The most important benefit for these natural metabolites is their enrolment amongst the health promoting constituents of natural foods which contains them. The European Foods Safety Authority (EFSA) recommends consuming about 1.5 - 2.4 g/day of phytosterols and/or stanols in order to reduce blood cholesterol [2]. Furthermore, FDA has approved the role of foods containing phytosterol esters inside a low saturated fat and cholesterol diet in reducing the risk of heart disease, especially consumption of at least 1.3 g/day sterols, twice a day [3]. The natural foods and high phytosterol-containing dietary has been continuously marketed for decades in diverse countries. Vegetable oils and products made from them, nuts, cereal products, vegetables, fruit and berries have been classified as richest or significantly rich sources of phytosterols [4]. Three phytosterols including β-sitosterol, campesterol and stigmasterol (Figure 1) are predominant sterols in the human herbal nutrition forming 65%, 30% and 3% of diet contents, respectively [5]. Phytosterols, with a long history of consumption as food or pharmaceutical products, have generally recognized as safe (GRAS), and no undesirable side effects have been reported. An exception is an illness named “phytosterolaemia”, a genetically disease, related to some mutations in the ABCG5/G8 proteins which play the role of protein pump to enter the sterols into enterocytes and hepatocytes [6, 7].

Fig.1: Chemical structures of some phytosterols.



2. SYNTHESIS OF B-SITOSTEROL

Although β-sitosterol has not been completely synthesized so far, it has been produced from pure stigmasterol via two ways. In the first rout, the side chain Δ22–23 alkene is selectively hydrogenated to produce β-sitosterol together with diverse levels of stigmasterol and fully saturated stigmastanol, while this selective hydrogenation accompanied by protection of Δ5–6 alkene to cyclopropyl carbinyl ether is purposed in the second approach. This process should follow by hydrogenation of the Δ22–23 double bond and also solvolysation of the cyclopropane in order to produce the C3-alcohol and Δ5–6 alkene again. The latter method seems very useful due to achievement of β-sitosterol in high purity. As a fact, semi-synthesis of β-sitosterol is still a challenge because of producing the methyl ether by products, whose removal is difficult [8, 9].

3. BIOSYNTHESIS OF B-SITOSTEROL

Biosynthesis of the phytosterols is regulating during membrane biogenesis. The literature showed that β-sitosterol is biologically synthesized from both mevalonate and deoxyxylulose pathways. Using 13C-labeling approach, the mechanism of β-sitosterol biosynthesis has been studied and although varies found according to the organism used, cycloarteol has been identified as an initial substrate. Actually, one molecule of isopentenyl diphosphate (IPP) joins to two molecules of dimethylallyl diphosphate (DMAPP) to produce farnesyl diphosphate diphosphate (FPP). Two of the later molecule (FPP) are then combined tail-to-tail to result in formation of squalene, as a triterpene and finally cycloartenol [10].

4. PHARMACOLOGICAL ACTIVITIES

4.1. Anti-inflammatory Activity

Prieto et al., (2006) reported the in vivo effect of β-sitosterol in a model of delayed-type hypersensitivity (DTH). They revealed that this compound can modulate a cell-mediated edema but it was not effective on the arachidonate pathway of intact cells and did not inhibit the leukocyte infiltration measured as myeloperoxidase activity in biopsies. They emphasized that its response to oxazolone might be due to a different pathway independent of interleukin-4. Moreover, β-sitosterol was not able to inhibit the cyclooxigenase (COX) pathway responsible for prostaglandin E2 (PGE2) synthesis [11].

In another study, Loizou et al., (2010) determined the activity of β-sitosterol (dose ranged: 0.1-200 μM) on the expression of vascular adhesion and intracellular adhesion molecule 1 employing ELISA, alongside the monocyte attachment (U937 cells) in tumor necrosis factor-alpha (TNF-alpha)-stimulated human aortic endothelial cells (HAECs) using adhesion assay. They concluded that β-sitosterol was able to inhibit both vascular adhesion and intracellular adhesion molecule 1 expression in TNF-alpha-stimulated HAEC. Moreover, this compound acts as an inhibitor on phosphorylation of NFkB [12]. In fact, β- sitosterol reduces the NFkB transcription factor activity in macrophage cells.

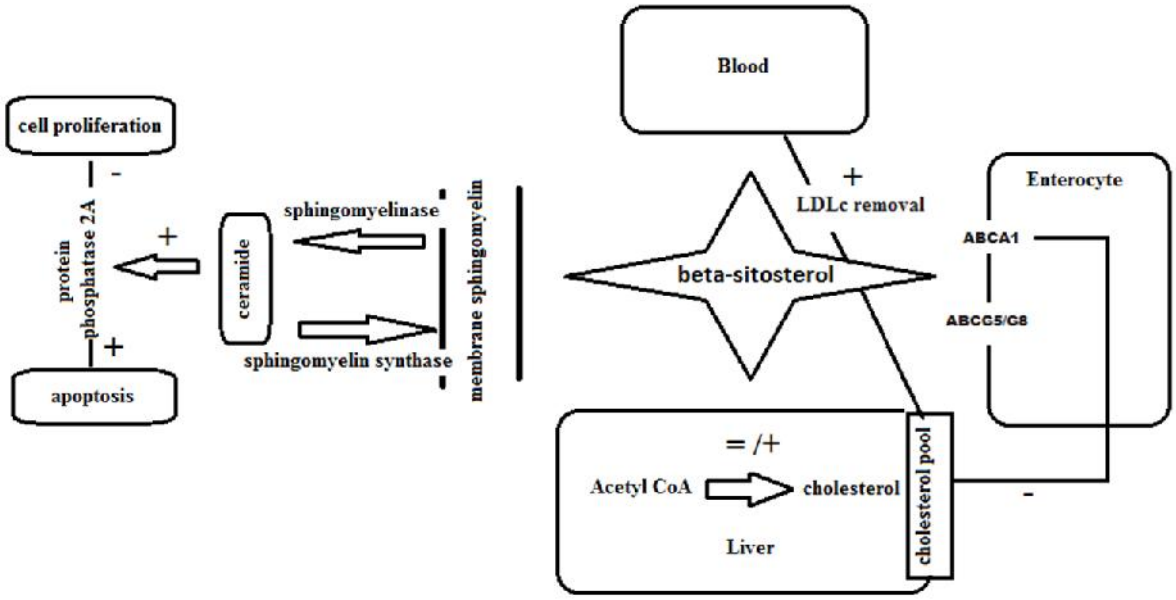
4.2. Inducing Apoptosis

Chai et al., (2008) reported that β-sitosterol could inhibit the proliferation of MCF-7 cells, in a dose-dependent manner. The above mentioned cell line was employed due to the presence of estrogenic receptors involving in breast cancer. The authors revealed a higher caspase activity (detected by increasing of DEVDase activity) after adding β-sitosterol to the cell line, resulted in caspase-induced apoptosis [13].

4.3. Chemoprotective or Chemopreventive Effects

In a review published by Ovesna et al., (2004), they recorded the experimentally inhibition of colon and breast cancer development by taraxasterol and β-sitosterol. They stated that these compounds can affect different levels of tumor development, such as their inhibitory effects on creation, promotion and induction of cancerous cells, as well as inhibition of tumor cells invasion and metastasis [14]. A schematic diagram for simplifying its mode of action in anticancer activity is shown in Figure 2.

Fig.2: Schematic diagram of anticancer activity of β-sitosterol.



4.4. Hypocholesterolemic Activity

Sugano et al., (1977), compared the hypocholesterolemic activity of β-sitosterol and its hydrogenated product, β-sitostanol (Figure1) in young male rats. They demonstrated that although hypocholesterolemic activity of sitostanol was significantly greater than sitosterol, their both effects on liver concentration of cholesterol and triglyceride were similar. Furthermore, sitostanol exhibited a high plasma triglyceride. Just apposite of sitostanol, sitosterol was decomposed by mean fecal recovery around 85% - 92%. The authors concluded that hydorgenation of plant sterols is a new achievement, because it would improve their hypocholesterolemic activity without affection on their safety regarding to initial sterols [15].

4.5. Angiogenic Effect

Angiogenesis is a noteworthy mechanism for wound healing activity of Aloe vera gel. It is demonstrated that its extracts exhibited an angiogenic activity on the chorioallantoic membrane (CAM) of chick embryo. β-sitosterol, recognized as the main compound of this gel, exhibited strong angiogenic effects in the CAM assay. This approach was obtained using neovascular stimulation in the mouse Matrigel plug examination and detection of human endothelial cells motility in an in vitro wound migration bioassay [16].

4.6. Genotoxicity Effect

Genotoxic assays are using to determine how much hurts is sustained in DNA by xenobiotics, which consequently may influence on human exposed to them. Paniagua-Perez

and co-authors (2005) reported the genotoxicity of β-sitosterol including the acute toxicity assay, which showed low lethal potential (38%) of this compound. The results indicated that no SCE (sister chromatid exchanges) increase was induced by tested doses (200, 400, 600, and 1000 mg/kg), as well as no changes in the cellular proliferation kinetics, or in the mitotic index. In that report, the highest applied dose showed 80% of the LD50. For this reason, β-sitosterol is not considered as genotoxic and/or cytotoxic. The safety of this compound encourages the scientists to perform more pharmacological investigations on this sterol [17].

4.7. Analgesic Bioassay

Villasenor et al., (2002) reported that the number of writhes for some fractions of Mentha cordifolia was decreased. This observation was recorded in levels comparable to the positive standard, mefenamic acid. They found that both β-sitosterol and its glucoside decreased the number of squirms (70% and 73% for each compound, respectively), which were induced using acetic acid [18].

4.8. Anthelminthic and Anti-mutagenic Activities

The above mentioned researchers have also reported β-sitosterol as an anthelminthic constituent of M. cordifolia. They employed in vitro tests by Ascaris suum, which resulted in the similar behavior of worms treated with β-sitosterol alongside the positive controls, combantrin and antiox. They claimed that β-sitosterol (by 0.5 mg /kg mouse administration), indicated anti-mutagenic activity and act as an inhibitor of tetracycline mutagenesis by 65.3%. Furthermore, administration of this compound alone, did not change the number of MN-PCE (micronucleated polychromatic erythrocytes) regarding to the control but differed from tetracycline [18].

4.9. Immunomodulatory Activity

Extremely little doses of β-sitosterol and daucosterol (its 3-O-D-glucoside) have been reported to elevate the in vitro proliferative activity of T-lymphocytes, when they were stimulated by phytohaemagglutinin (PHA) in the lower concentrations than optimum. Essential sterolin formulation (ESF) caused a significant augmentation in the expression of CD25 and HLA-Dr antigens on T- lymphocytes and also a growth in the secretion of IL-2 and gamma interferon. Either β-sitosterol or daucosterol increased the activity of NK-cells, while ESF showed a higher activity [19].

4.10. Effect on Benign Prostatic Hyperplasia (BPH)

In a systematic review by Wilt et al., (1999), four double-blind clinical trials were reported with lasting around 4-26 weeks. β-sitosterol alone was administered in three trials and a formulation of daucosterol (its glucoside) was consumed in another study. They concluded that β-sitosterol could improve the urinary symptom and flow in comparison of placebo, and did not decrease prostate size. In only trial with pure daucosterol no improvement in urinary flow was observed. Moreover, men who consumed β-sitosterol alone did not show different withdrawal rates from placebo. However, the duration of those studies was short and for this reason, probably effect of β-sitosterol in elongated period, its safety and capacity to prevent the complications of BPH are still in doubt [20].

4.11. Prostatic Cancer Treatment

In a study by Jourdain et al., (2006) the effect of several cocoa extracts, containing each of polyphenols or β-sitosterol, on two human prostate cancer cell lines (nonmetastatic and metastatic) as well as one normal cell line has been determined. The results revealed that cocoa extracts with polyphenol alone exhibited a potent and rapid reduction on cell growth compared to those contained β-sitosterol alone. They reported neither synergism nor additional activity by adding β-sitosterol to the cocoa polyphenols extract [21].

Another study undertaken by von Holtz et al., (1998) investigated the activity of two nutritional sterols (β-Sitosterol from herbal sources and cholesterol from animals) on prostate cancer cells regarding to evaluation of cell growth, differentiation, apoptosis, and sphingomyelin cycle intermediates. A decrease in cell growth (24%) and induction of apoptosis (fourfold) followed by cell rounding, also an enhancement in ceramide production (Figure 2) was considered by β-Sitosterol (16 μM). Cell differentiation (evaluated by prostate-specific antigen and prostatic acid phosphatase) showed no alteration, nevertheless total acid phosphatase was elevated by treating for one week. The researchers suggested that those observations have been created by activating the sphingomyelin cycle [22].

5. DISTRIBUTION OF B-SITOSTEROL IN PLANTS AND ALGAE

β-sitosterol is an ancient molecules in plants kingdom. Simple sterols have evolved into more complex forms from single cellular organisms to vascular plants. As shown in the literature, fungi, algae and protozoa, synthesize 24β- methyl sterols or ergosterols, while plants synthesize 24 α- ethyl sterols like sitosterols [23]. Literature review revealed that β-sitosterol has been isolated and purified by different chromatographic methods from diverse plant families. Some important plant and marine sources of this compound have been summarized in Table 1. Distribution of this compound and its derived components consists of a wide range of plant families, and the plants discussed here are just some well-known sources.

Table 1. Some important plant and marine sources of β-sitosterol and/or its glucoside and/or esters.

Plant family	Sources	Reference
Lamiaceae (Labiatae)	*Hymenocrather calycinus*	[24]
	Salvia hypoleuca	[25]
	Salvia macrosiphon	[26]
	Salvia limbata	[27]
	Satureja khuzistanica	[28]
	Satureja macrantha	[29]
	Satureja sahendica	[30]
	Satureja spicigera	[31]
	Satureja bachtiarica	[32]
	Lagichilus cabulicus	[33]

	Dracocephalum kotschyi	[34]
Asteraceae (Compositae)	*Achillea talagonica*	[35]
	Achillea tenuifolia	[36]
Apiaceae (Umbelliferae)	*Lomatopodium staurophyllum*	[37]
	Ferulago subvelutina	[38]
Rosaceae	*Geum iranicum*	[39]
Rubiaceae	*Knoxia valerianoids*	[40]
Fabaceae (Leguminisae)	*Tephrosia purpurea*	[41]
	Tephrosia uniflora.	[42]
	Tephrosia candida	[43]
Gracilariaceae (marine algae)	*Gracilariopsis persica*	[44]
	Gracilaria salicornia	[45]
Zingiberaceae	*Alpinia galangal*	[46]
Tiliaceae	*Tilia Americana*	[47]
Verbenaceae	*Lippia nodiflora**	[48]
Cucurbitaceae	*Momordica charantia*	[49]
	Coccinia indica	[50]
Solanaceae	*Solanum Xanthocarpum*	[51]
	Lycium chinensis	[52]
Thymelaeaceae	*Thymelea hirsute*	[53]
Acanthaceae	*Hygrophila spinosa*	[54]
Thymelaeaceae	*Aquilaria sinensis*	[55]

Glycine max (soybean, Fabaceae) is a valuable medicinal food plant and well-known for high contents of its phytosterols. A nonepolar extract of G. max contains at least 13 main sterol components. The literature showed that C4-desmethyl Delta (5)-sterols (e.g. β-sitosterol) were the predominant sterols found in shoots of G. max, while cycloartenol and 24(28)-methylene cycloartanol were mainly compacted in seeds [56]. As it is revealed in the literature, the entrance of foods containing soy products in human nutrition decreases the risk of mortality and recurrence of breast and colorectal cancer especially during menopause in women [57, 58].

6. ISOLATION AND IDENTIFICATION OF B-SITOSTEROL FROM HERBAL EXTRACTS

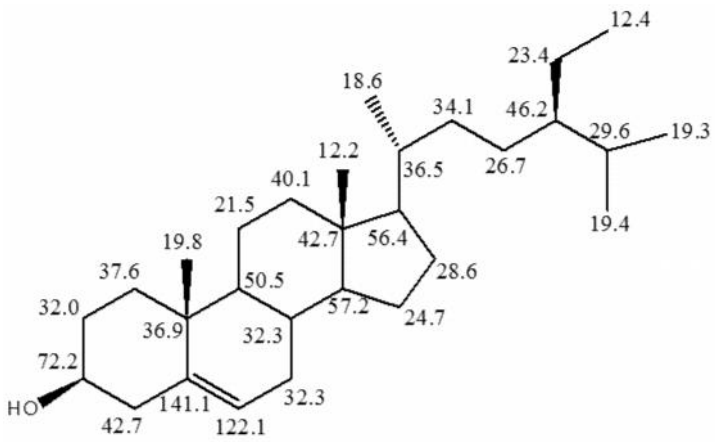
β-sitosterol is the dominant phytosterol, which may undergo oxidative process just like cholesterol, resulting in β-sitosterol oxides. This makes isolation of pure β-sitosterol a challenge due to presence of sitosterol oxides [59]. The common isolation procedure is preparing a chloroform extract from a plant, then performing various chromatographic separations on silica gel column and monitoring the fractions on TLC. Sometimes, the fraction containing β-sitosterol is dissolved in a mixture of chloroform: ethanol (2:3) followed

by heating on a water bath. Needle crystals might be appeared by leaving the solution undisturbed in a refrigerator [56].

HPLC with reverse phase stationary phase (RP-18) is one of the most applied chromatographic techniques for this purpose. Capillary gas chromatography-mass spectrometry (GC/MS) technique is also employed to determine either sitosterol oxides in vegetable oils or sterol esters [60, 61]. Moreover, plant sterols could be analyzed using high performance liquid chromatography-atmospheric pressure chemical ionization mass spectroscopy (HPLC-APCI-MS) [62]. Nevertheless β-sitosterol-D-glycoside is more polar than β-sitosterol itself, it has been reported to separate from petroleum ether fraction of Ocimum sanctum, as a yellow amorphous solid, soluble in petroleum ether, ethyl acetate, chloroform and dichloromethane [63]. When an herbal extract contains both β-sitosterol and stigmasterol, isolation of these similar analogs is not simple. However, β-sitosterol and stigmasterol are frequently isolated and purified from petroleum ether fraction of crude methanol extract via chromatography methods [64].

When the hexane or petroleum ether extract of a plant containing sterols subject to thin layer chromatography, using normal phase silica gel as stationary phase and petroleum ether: chloroform, hexane: ethyl acetate or chloroform: methanol as mobile phase, the chromatograms would show identical zones for steroidal nucleus with Liebermann - Buchard, vanillin – sulfuric acid or anisaldehyde- sulfuric acid visualizing reagents. Structural elucidation of β-sitosterol is commonly carried out by various spectral data from 1H- and 13C-NMR, IR and Mass spectroscopy, like other plant sterols. Concise data for 13C-NMR of β-sitosterol are indicated in Figure 3. This compound usually forms a white crystal with a melting point around 138 °C and also has no absorption under UV-Vis Lamp (254 and 366 nm), whereas its λ max in ethanol is at 206 nm and its main IR bands may appear at 3549 (OH), 2935 (CH2), 2867 (CH), 1637 (C=C) and finally 1063 (C-O), (all absorptions are in cm-1). High-resolution Mass spectra of β-sitosterol confirm its molecular mass at m/z: 414.7, which would be related to the molecular formula C29H50O. Characteristic fragments observed in EI-Mass are at m/z: 414, 396, 381, 329, 289, 273, 255, 213, 199 and 173. NMR spectrum of this compound shows the presence of six methyl groups, eleven methylene and three quaternary carbons together with a hydroxyl group. The olephinic carbons are appeared at 140.7 (C-5) and 121.7 (C-6) ppm. The number of carbons, extracted from ^{13}C-NMR, may reveal the structure of a sterol with 27 carbons (Figure 3). Comparison of the experimental data with those reported in the literature supports the proposed structure of this compound (Figure 3) [65-70].

Fig.3: Chemical shifts of ^{13}C-NMR in the structure of β-sitosterol.



7. OTHER BIOLOGICAL ACTIVITIES OF B-SITOSTEROL

Abdul Rahuman et al., (2008) reported the moderate larvicidal activity of five herbal medicines including: Jatropha gossypifolia, Abutilon indicum, Aegle marmelos, Euphorbia thymifolia, and Solanum torvum on the larvae of Culex quinquefasciatus. Interestingly, the main isolated compound from the petroleum ether extract of A. indicum (as the most active plant) was identified as β-sitosterol, which introduces this natural compound as a novel mosquito larvicidal sterol [71].

In another study by Gomes et al., (2007), β-sitosterol is reported as a neutralizing agent on viper and cobra venom. First, they found this activity in methanol extract of the roots of Pluchea indica (Asteraceae), growing wildly in India, then they isolated a mixture of β-sitosterol and stigmasterol (low percentage) using bioactivity guided fractionation. The authors followed anti-snake venom activity by study on experimental animals, which revealed the possible mechanism of action via antagonizing venom-induced changes in lipid peroxidation and superoxide dismutase activity [72].

Moreover the above mentioned effects, β -sitosterol is found as an antibacterial and antifungal agent separated from methanol extract of Senecio lyratus belonging to Asteraceae family [73]. Antimicrobial activity of pure β- sitosterol has been also reported using agar disk diffusion method. The authors claimed antibacterial activity ranged 10-14 mm for E. coli, P. aeruginosa, S. aureus and K. pneumonia, approximately equal to the standard Gentamicin [49]. Antimicrobial activity of β –sitosterol is still in doubt, because there are some reports which did not demonstrate such an activity from this compound alongside the reported active components [24, 74]. For instance, antimicrobial activity of β–sitosterol and β-sitosterol-3-O-D-glucoside has been evaluated on S. aureus, B. subtilis, E. coli, P. aeruginosa and two fungi, A. niger and C. albicans, leading to no effects with MICs above 200 µg/ml [75].

Furthermore, Nweze et al., (2011) reported an in vitro trypanocidal activity from seeds of Buchholzia coriacea (Capparaceae family), which has been applied for fever in African folklore medicine. The authors claimed that β-sitosterol was the active components against bloodstream forms of Trypanosoma brucei brucei S427 [76]. Although trypanocidal activity of β-sitosterol was observed against T. brucei (the causative agent of African Trypanosomiasis or sleeping sickness), we did not find it effective against epimastigotes of T. cruzi, the etiological agent of American Trypanosomiasis or Chagas disease [34].

As previously stated here, β-sitosterol as well as other phytosterols act through multiple modes of action, including inhibition of cancer-cell growth, angiogenesis, invasion and metastasis, and also by promoting apoptosis in cancerous cells. Literature showed that SinnolZym is a potent anti-cancer drug consisting of an adaptogenic mixture of the fermented herbal compounds (two strong phytosterols, Cerulin and Zorvan) which are separated from well-known medicinal plants. Capsaicin (the main bitter component of Capsicum spp.) is reported to decrease the anti-cancer activity of SinnolZym. The activity of Capsaicin is mediated by vanilloid receptors and promoting the release of a protein called substance P, which causes pain and inflammation [87].

9. OTHER ANALOGUES OF B-SITOSTEROL

So far, more than 250 various phytosterols and related derivatives have been found in diverse plants and marines, divided into three sub-groups as: 4-desmethyl sterols, 4α-monomethyl sterols, and 4,4-dimethyl-sterols, of which two later classes are less identified than 4-desmethyl sterols [88]. β-sitosterol, campesterol, and stigmasterol are the most existed phytosterols belong to the group of 4-desmethyl sterols. These compounds may find in the form of aceate, glucoside, oleate and linoleate esters, and also methyl and ethyl ethers. Even though β-sitosterol is well-known bioactive plant sterol, pharmacological and biological activities are reported from other related analogues in animals and human. Table 3, show a summary of diverse activities from these compounds.

Table 3. Biological and Pharmacological activities of other analogues of β-sitosterol.

Type of phytosterol	Activities	REFERENCES
Daucosterol (β-sitosterol-3-O-D-glucoside)	Protection of mice against candidiasis by the CD4+ Th1 immune response.	[89]
Stigmasterol	Inhibition of various pro-inflammatory degradation mediators involving in osteoarthritic-induced cartilage degradation, possible mechanism: inhibition of the NF-kappaB pathway.	[90]
	Significant antihypercholesterolemic activity without adverse effect on heart and liver. Inhibition of cholesterol absorption (54%). High doses (up to 52 mg/day) enhanced cholesterol, coprostanol and bile acid output.	[91]
	Inhibition of hepatic synthesis and intestinal absorption of cholesterol in the rat.	[92]
	Ameliorating effect on scopolamine-induced memory	[93]
		[94]
		[95]
Campesterol	Anti-angiogenic activity by inhibition of endothelial cell proliferation and capillary differentiation.	[96]
	Decrease the biliary secretion in compared with cholesterol.	[97]

Fucosterol	Antioxidant and hepatoprotective activities in rats were observed. Inhibition of histamine (97%) and acetylcholine (94%) induced contractions. Anti-diabetic activity: administration of fucosterol (30 mg/Kg in streptozotocin-induced diabetic rats) led to less serum glucose concentrations	[98] [99] [100]
Gorgosterol and its oxygenated analogues	Weak antifungal activity	[101]
Ergosterol	Increase sensitivity of cells to amphotericin B	[102]
Ergosterol peroxide	Antibacterial activity on *M. tuberculosis,* only with the Bactec 460 system	[103]

10. CONCLUSION

Phytosterols, found abundantly in none polar fractions of plants and marines, are consumed (200-400 mg daily) in human diets. Some of these compounds are structurally resembled cholesterol (such as β-sitosterol, stigmasterol and their analogues) and be able to inhibit the absorption of cholesterol, cancer-cell growth, angiogenesis, invasion and metastasis. Moreover, diverse biological activities are observed using these natural compounds or the extracts, in which implicated, e.g. antimicrobial, trypanocidal, mosquito larvicidal and as neutralizing agent on viper and cobra venom. Among the above mentioned sterols, β-sitosterol is well-known natural sterol in composition of known herbal drugs for treatment of benign prostatic hyperplasia and prostat cancer. High potential of this compound and its analogues in treatment of various illnesses, classifies this compound as the noteworthy drug of the future, although its role in treatment of BPH is now approved via clinical trial confirmations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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CONSENT (WHERE EVER APPLICABLE)

No patient was involved in this study.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

No human or animal subjects were involved in this study.

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