

How to Cite:

Gowri, K., Chitra, V., & Dev Sudarsan, V. (2022). Effect of phytosterols on osteoarthritis studied using insilico and CAM assays. *International Journal of Health Sciences*, 6(S4), 7187–7201. <https://doi.org/10.53730/ijhs.v6nS4.10158>

Effect of phytosterols on osteoarthritis studied using insilico and CAM assays

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Abstract---Aim: The aim of the present study was to evaluate the anti-arthritis and anti-inflammatory effects of stigmasterol and β -sitosterol. Method: The CAM test was used to investigate the anti-arthritis and anti-inflammatory properties of stigmasterol and -sitosterol, which were obtained commercially. The anti-oxidant activity of the 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were measured in vitro. Using Autodock 4.2.6, in silico docking of plant sterol was performed to verify the binding effectiveness of the plantsterol with the receptor. The Chick chorioallantoic membrane (CAM) assay was studied for chondrogenesis in correlation to infiltration of inflammatory mediators in a lethality study of Brine Shrimp larvae in which the administration of test (Phytosterols) and standard (NaCl, NaOH) drugs was estimated. By estimating the toxicity of the phytosterols, the Chick chorioallantoic membrane (CAM) assay was studied for chondrogenesis. Results: The CAM experiment demonstrates that the mTORC1 signalling pathway has anti-arthritis and anti-inflammatory properties, as well as the ability to deactivate particular inflammatory mediators, resulting in increased cell recovery and joint health. In the CAM experiment in chick embryo, 10 g and 20 g of each phytosterol were used, respectively. The phytosterols had a toxicity LC50 of 87.7 g/ml, while the normal potassium dichromate had a toxicity LC50 of 1.99 g/ml. The CAM membrane was found to reduce inflammation in synovial joint cartilages at a greater concentration of 20 g/ml. When docked with Cyclooxygenase-2 and p38 MAP Kinase, Stigmasterol has

a higher binding energy than Beta-sitosterol. When docked with Arachidonate-12-lipoxygenase, Beta-sitosterol had a higher binding energy than Stigmasterol. Conclusion: From the study, Stigmasterol and β -sitosterol managed the inflammation and oxidative properties plays a significant role in human health care system.

Keywords---osteoarthritis, stigmasterol, β -sitosterol, inflammation, anti-oxidant.

Introduction

Osteoarthritis is a chronic degenerative disease of synovial joints in which gradual degradation of articular hyaline cartilages with proliferation of new bone in joints are observed. In general, the movement between the bones are done with the help of a rubbery material called cartilages. In this condition the articular cartilage completely or partially deteriorates and makes the two ends of the bones to rub on each other^(1,2,4,5). There are two types osteoarthritis, which is Primary osteoarthritis and Secondary Osteoarthritis. The Primary OA are considered to be an idiopathic disease and commonly seen in elders. The Secondary OA are due to common causes include, age, gender, obesity, joint injuries, bone deformities, genetics etc. There is no cure to this disease but presently only palliative treatments are available for this⁽²⁶⁾. The drugs given only reduces the pain and also to prevent any further complications associated with it like osteonecrosis, chondrolysis, haemorrhage inside the joints etc.

The drugs used for palliative treatment of OA are mainly DMRD's like methotrexate, hydroxychloroquine, NSAID's like ibuprofen, naproxen, counter irritants like capsaicin or menthol and also interleukin therapies are employed.⁽²⁾ Phytosterols are a class of bioactive compounds derived from plant materials like vegetables oils, soy bean etc which are known for its cholesterol lowering properties mainly, but it also has anti-inflammatory properties^(2,5)

Materials and Methods

In silico docking

Protein preparation

The crystal structure of target proteins p38 MAP Kinase [PDB ID: 1A9U], Cyclooxygenase-2 [PDB ID: 3LN1] and Arachidonate-12-lipoxygenase [PDB ID: 3D3L] were taken and complexes associated with the target proteins are removed. The flexible residues for each target are identified by using the Visual molecular dynamics tool. These flexible residues are the active binding sites of the targets to which the ligand molecule binds.

Ligand Preparation

The SDF format of the curcumin, beta-Sitosterol, Stigmasterol and campesterol were taken from pubChem and submitted to NCI smiles translator to generate

PDB format of the ligand molecule. The PDBQT format of ligand molecule required for docking was generated by using Autodock 4.2.6.

Anti oxidant activity

Determination of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay [1]

DPPH radical scavenging assay of test samples (Stigmasterol, β -Sitosterol) were performed as per the method defined by Perumal *et al.*, 2018. Briefing, 0.135 mM DPPH was prepared in methanol. Different concentrations of samples (5, 10, 20, 40, 80, 160 and 320 μ g/ml) were mixed with 2.5 ml of DPPH solution. The reaction mixture was vortexed thoroughly and kept for 30 min at room condition. The reference standard Ascorbic acid was used taking same concentrations as that of test sample. The Absorbance of the mixture was measured at 517 nm. The ability of test samples and control to scavenge DPPH radical was calculated by:

$$\% \text{ DPPH inhibition} = \frac{OD \text{ of control} - OD \text{ of test}}{OD \text{ of control}} \times 100$$

Determination of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical scavenging assay [1]

ABTS radical scavenging assay of test samples (Stigmasterol, β -Sitosterol) were performed according to the modified method of Perumal *et al.*, 2018. The ABTS (7 mM, 25 ml in deionized water) stock solution was prepared with potassium persulfate ($K_2S_2O_8$) (140 mM, 440 μ l). Different concentrations of (5, 10, 20, 40, 80, 160 and 320 μ g/ml) of test samples and standard (Ascorbic acid) were mixed with the ABTS working solution (2.0 ml) and the reaction mixture was kept for 20 min in room temperature. The Absorbance of the mixture was measured using an ultraviolet-visible spectrophotometer at 734 nm. The ABTS radical scavenging activity was given was calculated by the equation:

$$ABTS (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A0 is the control; A1 is the test

Brine shrimp lethality study

Lethality assay: Artificial sea water (ASW) was prepared using 38g of NaCl dissolved in 1L distilled water. In ASW brine shrimp egg *Artemia salina* was hatched maintaining the pH at 8.5 by 1N NaOH with constant aeration for 48hr under 29°C light sources. After they hatched the active nauplii discharge from egg shells were taken using pipette and used for the assay [2]. Samples (Stigmasterol, β -Sitosterol) dissolved in methanol was diluted at varying concentrations (10, 20, 40, 60, 80 and 100 μ g/ml) and Potassium dichromate was served as a positive control dissolving in artificial sea water ranging from 0.1 to 0.9 μ g/ml concentrations [3]. ASW with methanol served as negative control. To each sample vial ten nauplii were transferred using pipette containing 4.5ml of ASW and 0.5

ml of samples and kept in room temperature for 24 hr and the surviving nauplii were counted. Nauplii lying at the bottom of the vial were considered death [4,5]. After 24hr the survival nauplii were counted and the percentage mortality was measured by the formula:

$$\% \text{ mortality} = \frac{\text{No of dead nauplii}}{\text{Initial no of live nauplii}} \times 100$$

Depending on the % mortality, the graph was plotted by taking Concentration Vs % mortality. The median lethal concentration (LC₅₀) of the test samples was determined by probit analysis as described by Finney for toxicity measurement [6].

In ovo chick chorioallantoic membrane (CAM) assay

Fertilized chicken egg *Gallus gallus domesticus* was collected from the local area sterilised by 50% alcohol, numbering and incubated in aseptic incubator at 37°C with 70-75% humidity. On day 3, albumen of 2-3 ml was taken out from a window in the air sac and closed with sterilised parafilm tape where the eggs again were kept inside the incubator. On day 9, four groups contained and the dose of the samples (Stigmasterol, β -Sitosterol) of (10,20 μ g/ml) was selected based on mortality studies of brine shrimp, sham control (PBS), positive control (NaOH) and a negative control (NaCl) installed with 0.3ml, on day 12 all eggs were observed and removed from the shell. Histological evaluation was carried out by fixing in 10% formalin embedded in paraffin and 6 μ m serial section was cut further processed for stained preparation of haematoxylin- eosin and examined under light photomicroscope. [7,8,9]

Results

Beta-sitosterol with Cyclooxygenase-2 [3LN1]

Beta-sitosterol is docked with Cyclooxygenase-2 and the binding energy score is - 5.04 Kcal/mole. The inhibitory constant for this docking is 200.48 μ M. GLN 189, HIS 193, THR 198, ASN 368, HIS 372 and TYR 371 are the flexible residues involved in the docking. HIS 372 is the residue involved in the formation of the hydrogen bond with ligands. The desolv energy and electrostatic energy for this docking is zero.

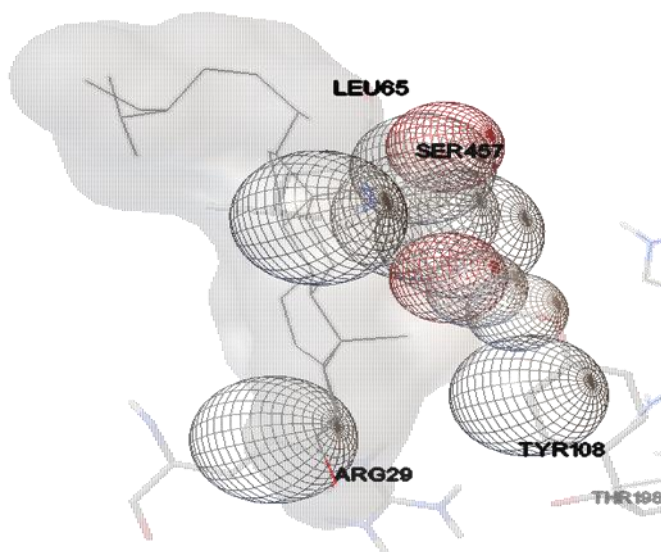


Figure 1: Beta-sitosterol with Cyclooxygenase-2 [3LN1]

Stigmasterol with Cyclooxygenase-2 [3LN1]

Stigmasterol is docked with the Cyclooxygenase-2 and the binding energy score is -7.12 Kcal/mole. The inhibitory constant for the docking is 6.04 μ M. There is no formation of hydrogen bond with the ligand. GLN 189, HIS 193, THR 198, ASN 368, HIS 372 and TYR 371 are the flexible residues involved in the docking. GLN 189, HIS 193, ASN 368 and HIS 372 are closely interacted with the ligand after the docking. The desolv energy is -3.1 Kcal/mole and the electrostatic energy is zero.

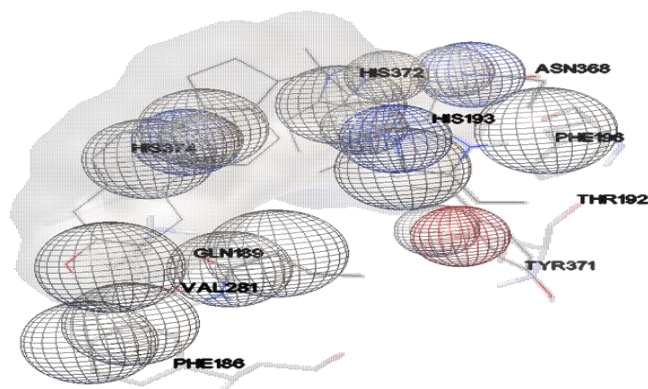


Figure 2Cyclooxygenase-2 [3LN1]

Beta-Sitosterol with p38 MAP Kinase [1A9U]

Beta-sitosterol is docked with the p38 MAP Kinase and the binding energy score is -6.73 Kcal/mole. The inhibitory constant for the docking is 11.66 μ M. The flexible residues involved are TYR 35, LYS 53, GLU 71, THR 106, HIS 107, and

ARG 173. There is no formation of hydrogen bond with the ligand. LYS 53, GLU 71 and THR 106 are the residues that are closely involved in the interaction with the ligand. -2.58 Kcal/ mole is the desolv energy observed in the docking. The electrostatic energy of this docking is -0.1 Kcal/ mole.

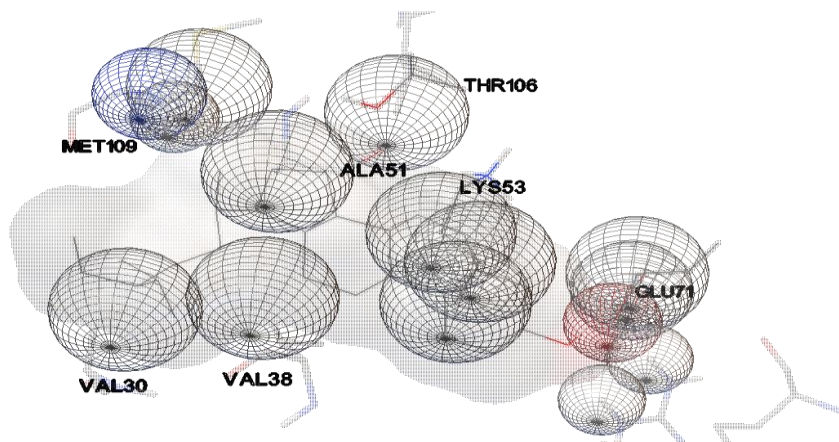


Figure 3: Beta-Sitosterol with p38 MAP Kinase [1A9U]

Stigmasterol with p38 MAP Kinase [1A9U]

Stigmasterol is docked with the p38 MAP Kinase to check the activity. The binding energy score found is -7.1 Kcal/ mole. The inhibitory constant for this docking is 11.66uM. TYR 35, LYS 53, GLU 71, THR 106, HIS 107, and ARG 173 are the flexible residues involved in the docking with the ligand. There is no formation of hydrogen with the ligand. TYR 35, LYS 53 and ARG 173 are the residues involved in docking and closely interacting with the ligand. The desolv energy for this docking is -3.8 Kcal/ mole and the electrostatic energy is -0.03 Kcal/ mole.

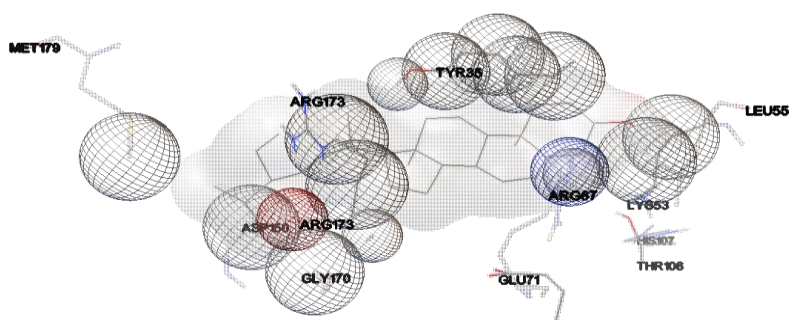


Figure 1: Stigmasterol with p38 MAP Kinase [1A9U]

Beta - sitosterol with Arachidonate-12-lipoxygenase [3D3L]

Beta - sitosterol docked with Arachidonate-12-lipoxygenase and the binding score is -8.61 Kcal/mole. The flexible residues involved in the docking are HIS 360, THR 364, HIS 365, HIS 540, ASN 544 and THR 662. Except THR 364 and HIS 540 remaining all the residues are involved in the interaction with ligand. HIS 360, THR 364 and THR 662 are involved in the formation of hydrogen bond with the ligand. The desolv energy and the electrostatic energy for this docking are -2.82 Kcal/ mole and 0 Kcal/ mole.

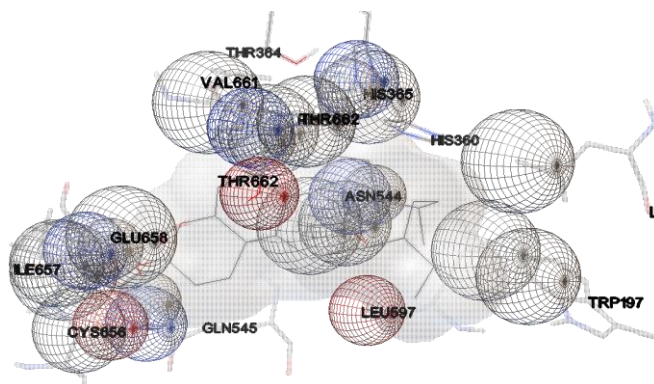


Figure 2: Beta - sitosterol with Arachidonate-12-lipoxygenase [3D3L]

Stigmasterol with Arachidonate-12-lipoxygenase [3D3L]

The binding energy score for the docking between Arachidonate-12-lipoxygenase and stigmasterol is -6.51 Kcal /mole. The inhibitory constant for the same docking is 16.77 uM. HIS 360, THR 364, HIS 365, HIS 540, ASN 544 and THR 662 are the flexible residues involved in the docking. All these residues are involved in the interaction with the ligand after the docking. The desolv energy and the electrostatic energy of this docking is -3.47 Kcal/ mole and 0 Kcal/ mole.

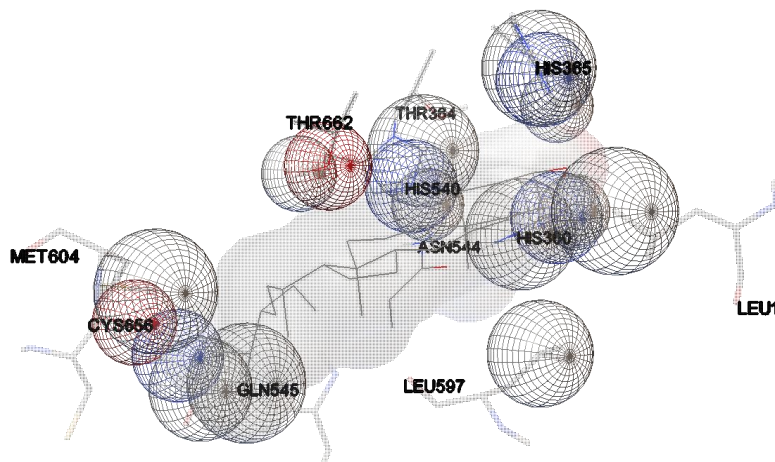


Figure 3: Stigmasterol with Arachidonate-12-lipoxygenase [3D3L]

Table : 1 Docking scores

S.No	Target	Target PDB ID	Ligand	Binding Energy Kcal/mole	Inhibitory Constant	H – Bonding	Flexible Residues Involved in Docking
1.	Cyclooxygenase-2	3LN1	beta-Sitosterol	-5.04	200.48	HIS372	GLN189 HIS193 THR198 ASN368 HIS372 TYR371
2.	Cyclooxygenase-2	3LN1	Stigmasterol	-7.12	6.04 uM	NO H-Bond	GLN189 HIS193 THR198 ASN368 HIS372 TYR371
3.	p38 MAP Kinase	1A9U	beta-Sitosterol	-6.73	11.66um	No H-bond	TYR35 LYS53 GLU71 THR106 HIS107 ARG173
4.	p38 MAP Kinase	1A9U	Stigmasterol	-7.1	6.25 uM	NO H-Bond	TYR35 LYS53 GLU71 THR106 HIS107 ARG173
5.	Arachidonate-12-lipoxygenase	3D3L	beta-Sitosterol	-8.61	489.48nM	HIS360 HR364T HR662	HIS360 HIS365 THR364 HIS540 ASN544 THR662
6.	Arachidonate-12-lipoxygenase	3D3L	Stigmasterol	-6.51	16.77 uM	THR364 THR662	HIS360 HIS365 THR364 HIS540 ASN544 THR662

DPPH radical scavenging assay

Anti-oxidant activity was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, IC₅₀ values of the test samples (Stigmasterol, β -

Sitosterol) and the standard drug (Ascorbic acid) determined by regression analysis found to be $>320 \mu\text{g/ml}$, $>320 \mu\text{g/ml}$ and $21.05 \mu\text{g/ml}$ respectively.

ABTS radical scavenging assay

ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] radical scavenging assay IC_{50} values of the test sample (Stigmasterol, β -Sitosterol) and the standard drug (Ascorbic acid) was determined to be $>320 \mu\text{g/ml}$, $>320 \mu\text{g/ml}$ and $20.86 \mu\text{g/ml}$ respectively.

Table 2: IC_{50} values of *In-vitro* antioxidant activity

Assay	Compounds	IC_{50} value ($\mu\text{g/ml}$)
DPPH	Stigmasterol	>320
	β -Sitosterol	>320
	Ascorbic acid	21.05
ABTS	Stigmasterol	>320
	β -Sitosterol	>320
	Ascorbic acid	20.86

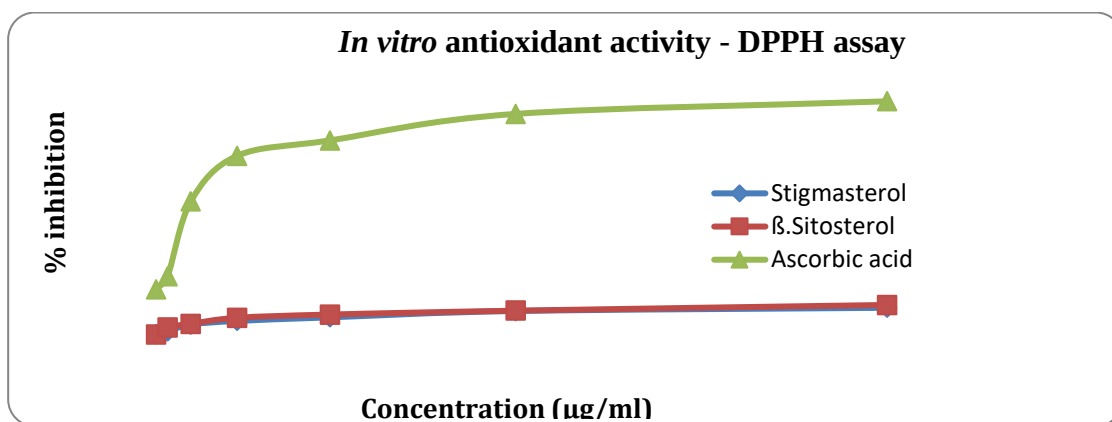


Figure 4: DPPH Assay

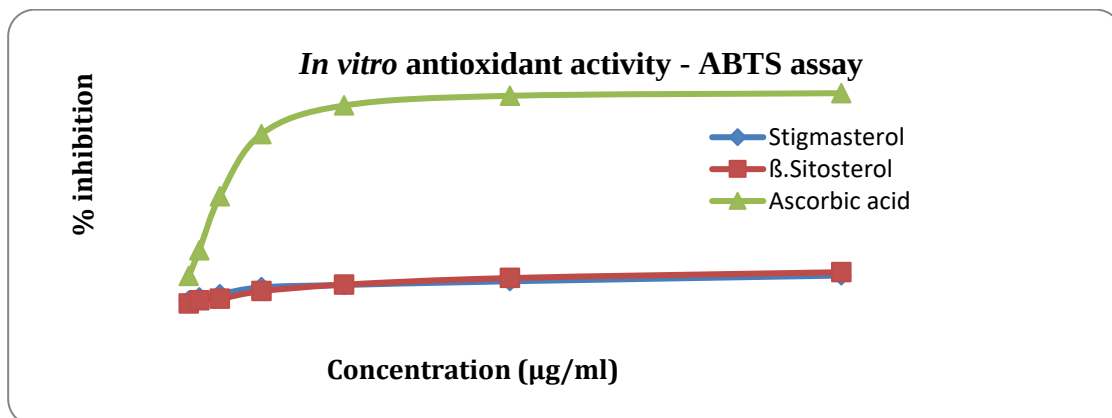


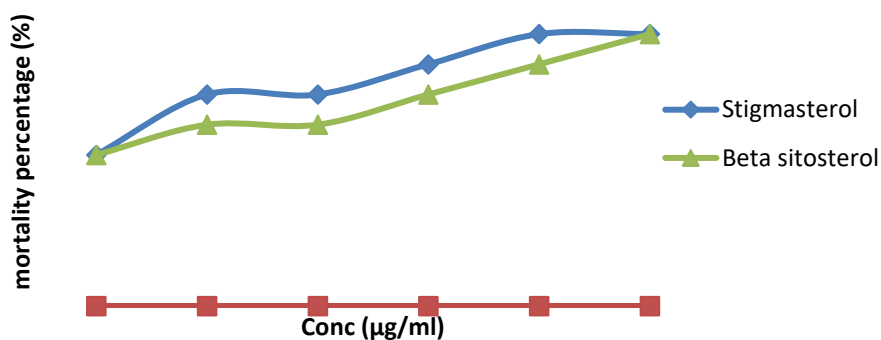
Figure 5: ABTS Assay

Brine shrimp lethality assay

The samples toxicity observed at higher concentration declared greater mortality rate, mortality percentages Vs log of Concentration was plotted and the toxicity was expressed as LC_{50} value. LC_{50} value for test is 87.71 $\mu\text{g/ml}$ and 1.99 $\mu\text{g/ml}$ for standard.

Sl no	Sample	LC_{50} ($\mu\text{g/ml}$)
1	Stigmasterol	87.71
2	β -Sitosterol	31.16
3	Potassium dichromate	1.99

Conc Vs %mortality for 24hr of stigmasterol and beta-sitosterol



Conc Vs %mortality for 24hr of potassium dichromate

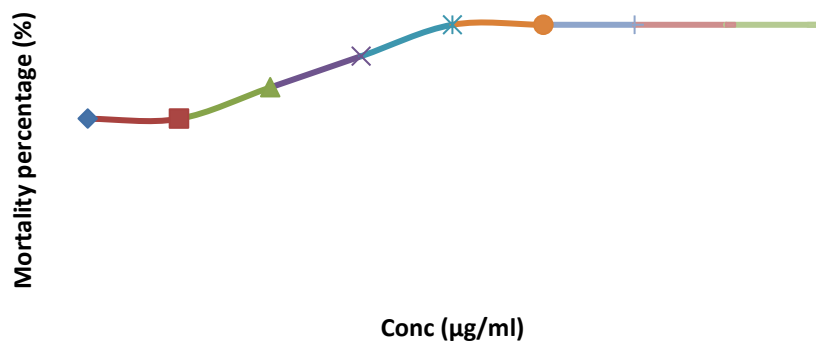
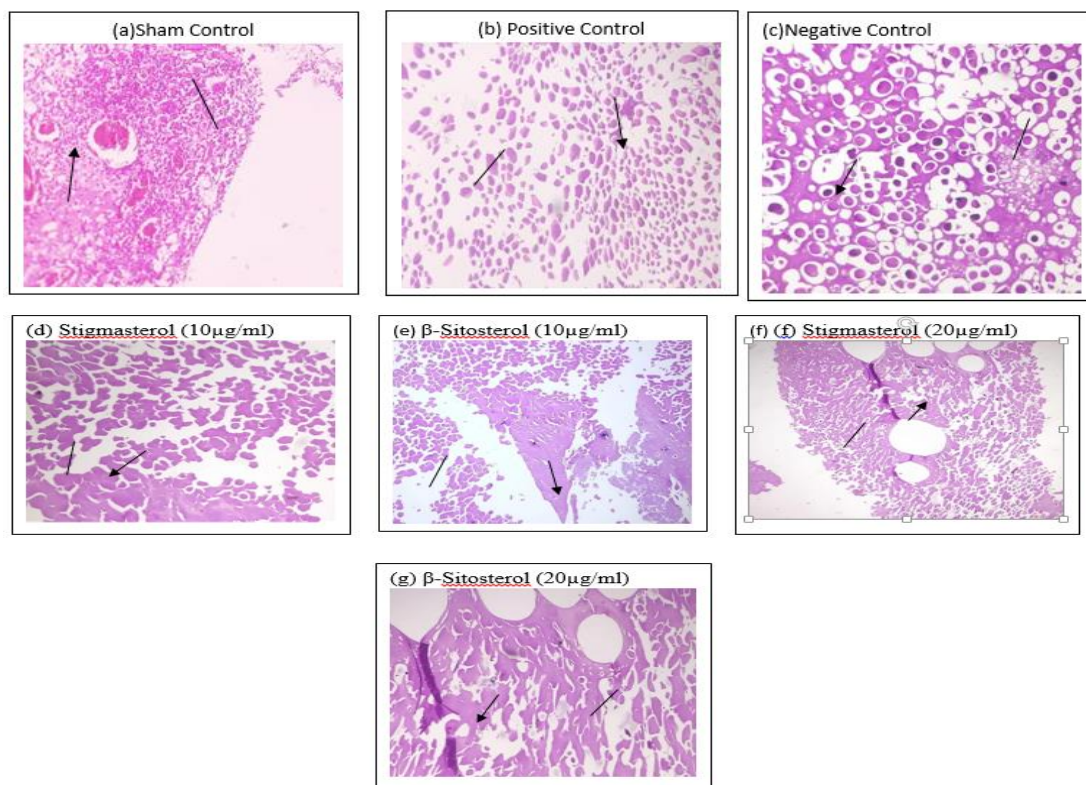


Figure 6: Graphical representation of lethality study

Chick chorioallantoic membrane (CAM) assay

The toxicity measurement mainly recognised in Chick chorioallantoic membrane (CAM) where the histopathological studies of sham control and negative control introduced no significant changes, less impairment of synovial joint cause joint inflammable observed at positive control. In tested group at low dose (10 μ g/ml) significant changes appeared as the synovial joints become inflamed but at a higher dose (20 μ g/ml) of Stigmasterol and β -Sitosterol it was observed in inhibiting the growth of synovitis hyperplasia and infiltration of inflammation.

Fig10: Histological evaluation on sham control, positive and negative control, stigmasterol and β -Sitosterol of 10 and 20 μ g/ml. A line designated as blood vessel, arrow designated fibroblast cell



Discussion

The goal of this work was to find a probable mechanism of action for stigmasterol and -sitosterol's anti-arthritis and anti-inflammatory actions in chick embryos. Chondrogenesis is controlled by the mTORC1 pathway, and it happens whenever old chondrocytes are lost or injured as a result of any stress. Osteoarthritis is caused by a reduction or failure in this process, which can be healed by using phytosterols to regulate it (24,25,32). Phytosterols activate protein synthesis (m-

RNA cap) precursors SK6 and 4EB1 via inducing RHEB and releasing GTP for the mTORc1 pathway.

The phytosterols have anti-oxidant properties, according to the study. The DPPH radical scavenging assay is a stable free radical at room temperature that accepts an electron/proton radical to produce a stable molecule (25,31,34). In silico docking demonstrates that the phytosterol has a higher binding affinity for treating osteoarthritis. The colour of DPPH, which is normally employed as a substrate to test antioxidant activity, changes from purple to yellow. As a result, these phytosterols have free radical scavenging properties, lowering cell damage and inflammation while also inhibiting inflammatory mediators such as Interleukin, Tumor Necrosis Factor Alpha, and others. As a result, phytosterols may be useful in the treatment of osteoarthritis and other inflammatory conditions. Other phytosterol actions can be investigated further.

Conclusion

Anti-arthritic activity, anti-oxidant activity, and toxicity studies by CAM Assay have proven that stigmasterol and -sitosterol show a greater response of anti-inflammatory disorder correlates to arthritic activity, and the oxidative response was determined by stigmasterol and -sitosterol active response in comparison to standard solutions. The toxicity of phytosterols was investigated for cartilage cell chondrogenesis and inflammatory mediator infiltration into the CAM membrane. Stigmasterol and -sitosterol may suppress the role of inflammation in osteoarthritis. Inflammation has been identified as a common feature in numerous diseases, including the underlying cause of degenerative disorders like osteoarthritis, according to research. We may conclude from this study that stigmasterol and -sitosterol limit inflammatory effects by inhibiting the formation of the IL and TNF pathways, which may have an influence on osteoarthritis symptoms. As a result, phytosterols have been shown to have an important function in human health care, propelling natural product research to new heights.

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