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AN IN VITRO ANTI - INFLAMMATORY RESEARCH ON ETHANOLIC EXTRACT OF *SINDHUWAR (VITEX NEGUNDO)* AND *SLESHMATKA (CORDIA DICHOTOMA)*

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ABSTRACT –

Background- Most developing countries resort to medicinal plants for treating diseases, but few of these have scientific backing for their use. The aim of the study was to validate traditional use of *Sindhuwar (Shweta Nirgundi) & Sleshmataka* in treating inflammation and determine the mechanism of action. **Material and Methods** - The in vitro anti-inflammatory action has been determined via Effect of Protein Denaturation, Total Polyphenol, Carotenoid and Flavonoids. **Results–**Total phenolic and flavonoid contents were found higher in ethanolic extract of *Sleshmataka*. **Conclusion –** The result shows that ethanolic extract of *Sindhuwar* and *Sleshmataka* possesses substantial anti-inflammatory properties.

KEY WORDS – Vitex negundo, *Sindhuwar*, *Sleshmataka*, Cordia dichotoma, anti-inflammatory, *Vishaghna Mahakashya*, *Ayurveda*

INTRODUCTION –

Inflammatory disease is one of the major health problems worldwide. Inflammation is a fundamental response of virtually all multicellular organisms to infection and injury. Functions of the inflammatory response include detection and response to invading microbes and to cellular damage and repair of tissue injury[1]. Both deficiencies and deregulation of inflammation underlie many common and life-threatening clinical disorders including asthma or bronchial hyper reactivity, infection, sepsis and atherosclerotic disease, which all have a substantial societal impact. Inflammation is caused by injury to a living tissue. The primary indicators of inflammation are - pain (dolor), redness (rubor), heat (calor) and swelling (tumor) and loss of function (functio

laesa). When there is an injury to any part of the human body, the arterioles dilate [2-5]. This produce redness by increasing the blood circulation towards the injured tissue. As well, inflammation is associated with pain, and it involves in an increase of protein denaturation, an increase of vascular permeability, and membrane alteration, among others. Inflammation is also described as the body response to inactivate or eliminate the invading stimuli or organisms, to remove the irritants and set the stage for tissue repair, and the process is accelerated by the release of chemical mediators from injured cells or tissues and migrating cells. The migration of leukocytes from the venous systems to the site of damage, and the release of cytokines, are known to play a crucial role in the inflammatory response. These chemicals cause widening of blood capillaries (vasodilatation) and the permeability of the capillaries. This will lead to increased blood flow to the injured site. It involve a series of events in which the metabolism of arachidonic acid plays an important role. It is metabolized by the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A2, whereas the 5-lipoxygenase (5-LOX) pathway to eicosanoids and leukotrienes (LT's), which are known to act as chemical mediators in a variety of inflammatory events[6-8]. Currently available anti- inflammatory drugs block both enzyme activities and relief symptoms despite they have serious side effects. Therefore, it is essential to administer anti-inflammatory drugs with lesser side effects.

A revisit to *Ayurveda* classics points to the understanding of inflammation as a vascular and cellular reaction. According to *Ayurveda*, vitiation in the microcirculation channels, or *Srotodushti*, causes (a) excessive functioning (b) obstruction or insufficient activity (c) tumours or new growths (d) Movements in unnatural directions. The first inflammatory response is usually increased activity (due to vascularity) in the form of excessive exudates and protein release into the extra cellular matrix. At that point theirs is hindrance leading to alter within the rate of diffusion of nutrients, oxygen and wastes. The hampered diffusion leads to tumours, benign or malignant. Movement in unusual direction may be due to reverse osmosis as a result of electrolyte differences. All the clogging of the micro channels has been attributed to *Aama*, the toxic by-product of improper digestion. *Aama* is considered the pro-inflammatory waste and the chief contributor to *Srotodushti*. It is interesting to note that there have been suggestions to quantify and qualify *Aama* according to its description in *Ayurveda*, that the *Aama* status of a person in a very personalized form of treatment might really be regarded as a bio-marker for chronic inflammation leading to metabolic syndrome and cancer will perhaps pave the way for preventive oncology and prevention of lifestyle disorders[8].

Here, it is also to be noted that *Granthi* or tumour formation is considered as a form of inflammation in *Ayurveda.*

STUDY DRUGS –

Study Drugs	Latin name and Family	Pharmacological Actions	Chemical Constituents
Sindhuwar	Vitex negundo Fam.- Lamiaceae	Antioxidant Anti- Bacterial Anti-inflammatory Anti-Fungal	Vitamin-C, α-terpineol, carotene, flavonoids, betulinic acid, ursolic acid, β-sitosterol, p-hydroxybenzoic acid, vitexoside, negundin A, negundin B[7-8].
Sleshmataka	Cordio dichotoma Fam.- Boraginaceae	Anti-inflammatory Anti-Fungal & Anti-Bacterial Antioxidant	Allantoin, β-sitosterol ,flavonoids, terpenes and sterols,fattyb acids such as palmitic acid ,stearic acid[9-10] etc.,

MATERIAL AND METHODS –

The Methodology conducted for this study has been grouped in following sections-

- **Plant sample collection** –

- ***Cordia dichotoma* G.Forst.** –The Original Fruits and Bark of the plant has been collected from Herbal Garden,Shayampur, Nazibabad Road in the month of June 2022.
- ***Vitex negundo* Linn.** - The Original leaves, roots and bark of the plant has been collected from its Natural habitat, Chilla Range, Haridwar, Uttarakhand in the month of June 2022.

- **Identification and Authentication** – All the selected plants parts were identified and verified by the eminent experts of Dravyaguna Dept. at Rishikul Campus, Haridwar (UAU) with reference no. DG/RC/UAU/-127 dated on 17/11/22.
- **Extraction process[9]** – 5 g coarsely powdered air dried drug (mixture of *sindhuwar* leaves, root, bark & *sleshmataka* fruits, bark) was macerated with 100 ml of Alcohol of the specified strength in a closed flask for twenty-four hours. It was then continuously shaken for six hours using rotary shaker and allowed to stand for eighteen hours. The content was filtered using filter paper. The filtrate was transferred to a pre-weighed flat bottomed dish and evaporated to dryness on a water bath. Then the dish was kept in oven at 105º, to constant weight and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried drug.

- **In vitro anti-inflammatory parameters[10-11] –**

- **Effect of Protein Denaturation-** The reaction mixture (5 mL) `consisted of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL of extract (5, 10 and 15 % w/v Concentration) and the mixture was mixed, and was incubated in a water bath (37 ◦C) for 15 min, and then the reaction mixture was heated at 70^0C for 5 min. After cooling, the turbidity was measured at 660 nm

using a UV/VIS spectrometer. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition of denaturation} = 100 \times (1 - A_2/A_1)$$

A_1 = absorption of the control sample, and A_2 = absorption of the test sample.

- **Total Phenolic Content (TPC)** - The evaluation of the Total Phenolic Content (TPC) in each sample was carried by using Folin Ciocalteu's reagent and UV-visible spectrophotometry. 500 µL of each sample was taken, placed and shaken with Folin- Ciocalteu reagent (0.1 M, 500 µL), at 45°C for 10 min. After this time, Na_2CO_3 (0.5%, 500 µL) was added, and the mixture was allowed to stand for 30 min at room temperature. After incubation, it was read at 765 nm. The results were expressed as mg Total Phenols Gallic Acid Equivalent (TPGAE) and Total Phenols Tannic Acid Equivalent (TPTAE) per gram of yogurt (mg TPGAE/g, mg TPTAE/g). A calibration curve for GA and TA (0.1-1.0 mg/mL) was prepared and processed in the same way as the samples.

- **Total Flavonoid Content (TFC)** – For Flavonoid quantification, 500 µL of each sample was taken, mixed with $NaNO_2$ (1.5%, 500 µL) in a vortex and allowed to stand for 10 min. Then, $AlCl_3$ (3%, 1000 µL) was added, mixed for 2 minutes, 1000 µL NaOH 1N was added, allowed to stand for 2 minutes and read at 490 nm. Finally, a quercetin standard curve was performed. The result was expressed as mg quercetin per 100 g.

- **Total Carotenoid content**- To determine the total amount of carotenoids content, approximately 5 g of the samples and 3 g of celite 454 were weighed. For the carotenoid extraction, successive additions of 25 mL of acetone were made to obtain a paste, which was transferred into a sintered funnel (5 µm) coupled to a 250 mL Buchner flask and filtered under vacuum. This procedure was repeated three times or until the sample became colorless. The extract obtained was transferred to a 500 mL separatory funnel containing 40 mL of petroleum ether. The acetone was removed through the slow addition of ultrapure water to prevent emulsion formation. The aqueous phase was discarded. This procedure was repeated four times until no residual solvent remained. Then, the extract was transferred through a funnel to a 50 mL volumetric flask containing 15 g of anhydrous sodium sulphate.

OBBSERVATIONS AND RESULTS –

TABLE 1– Results Showing effect of Protein Denaturation –

Conc.	Absorbance			% Inhibition	
	Blank	*Sleshmataka*	*Sindhuwar*	*Sleshmataka*	*Sindhuwar*
-	0.695	-	-	-	-
5 % w/v	-	0.087	0.149	39.2	35.4
10 %w/v	-	0.167	0.135	47.4	44
15% w/v	-	0.236	0.195	54.1	50
20 %w/v		0.456	0.365	76.1	67

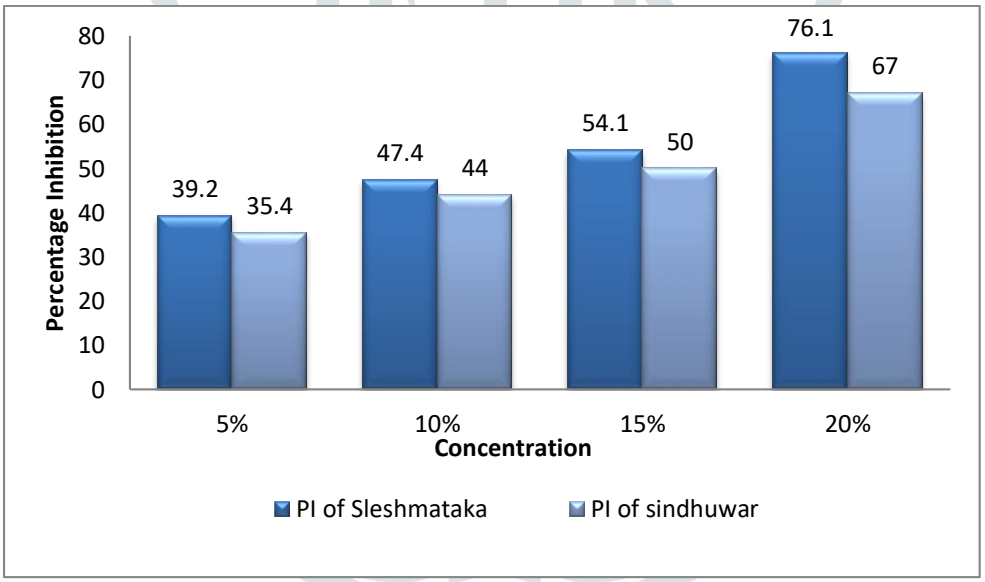
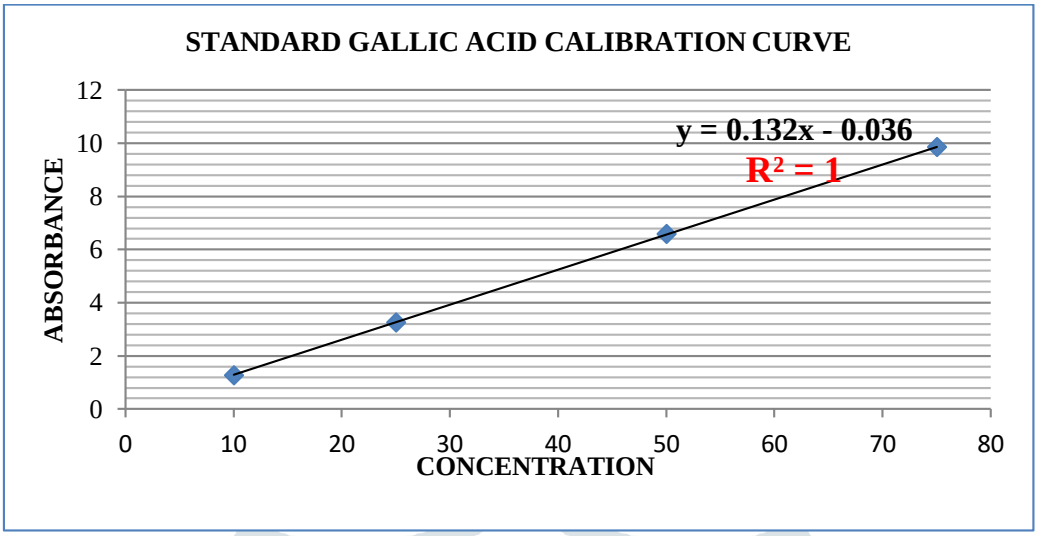


TABLE 2- Results showing Total Polyphenols content (TPC) –

Sample	**Concentration**	**Absorbance**
Gallic Acid	10 µg/ml	0.087
	25 µg/ml	0.167
	50 µg/ml	0.236
	75 µg/ml	0.456
Sleshmataka	200 µg/mL	0.695
Sindhuwar	200 µg/mL	0.236

The calibration curve was plotted using standard Gallic acid showing R- squared -



Calculation

Y=0.132X -0.036

Y= Absorbance of Extract

X= Concentration of Polyphenols

S. no	Test Sample	Polyphenol content (mg/GAE(g))
1	*Sleshmataka (200 µg/mL)*	5.538
2	*Sindhuwar (200 µg/mL)*	2.06

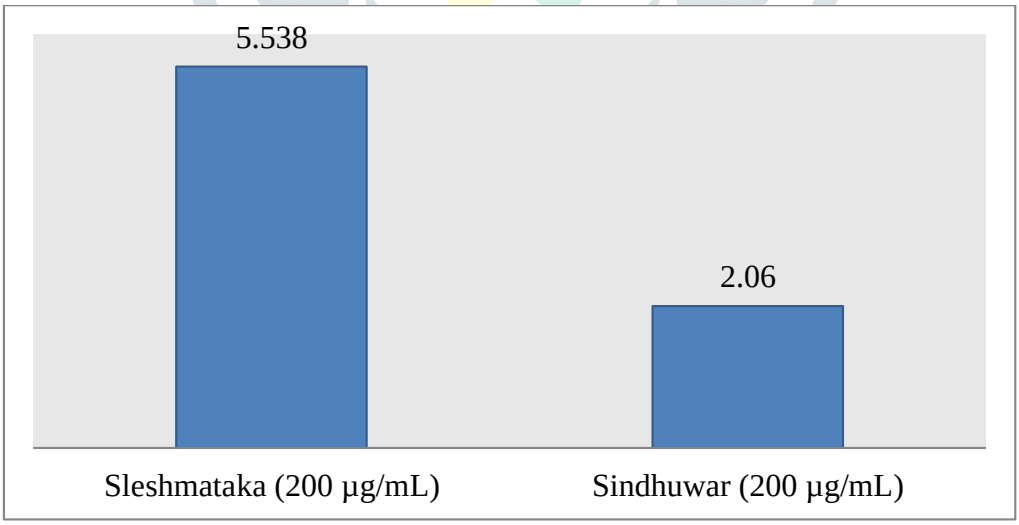
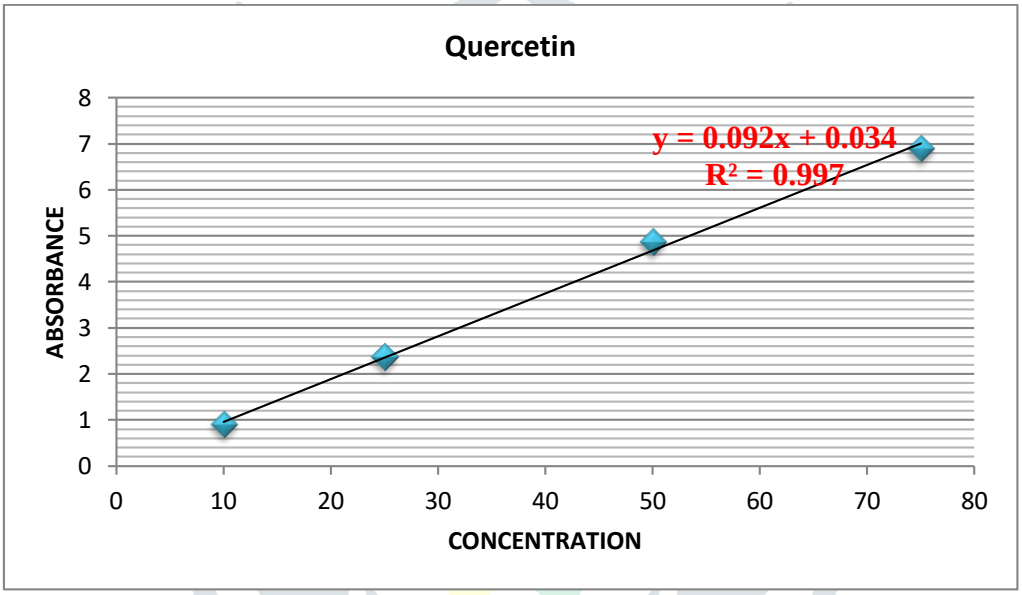


TABLE 3- Results showing Total flavonoid content (TFC)-

Sample	Concentration	Absorbance
Quercetin	10 µg/ml	0.895
	25 µg/ml	2.356
	50 µg/ml	4.857
	75 µg/ml	6.891
Sleshmataka	200 µg/mL	0.612
Sindhuwar	200 µg/mL	0.325

The calibration curve was plotted using standard quercetin showing R- square -



Calculation -

Y=0.092X +0.034

Y= Absorbance of Extract

X= Concentration of Polyphenols

S. no	Test Sample	Total flavonoid quercetin equivalent/ g)
1	***Sleshmataka (200 µg/mL)***	6.282
2	***Sindhuwar (200 µg/mL)***	3.163

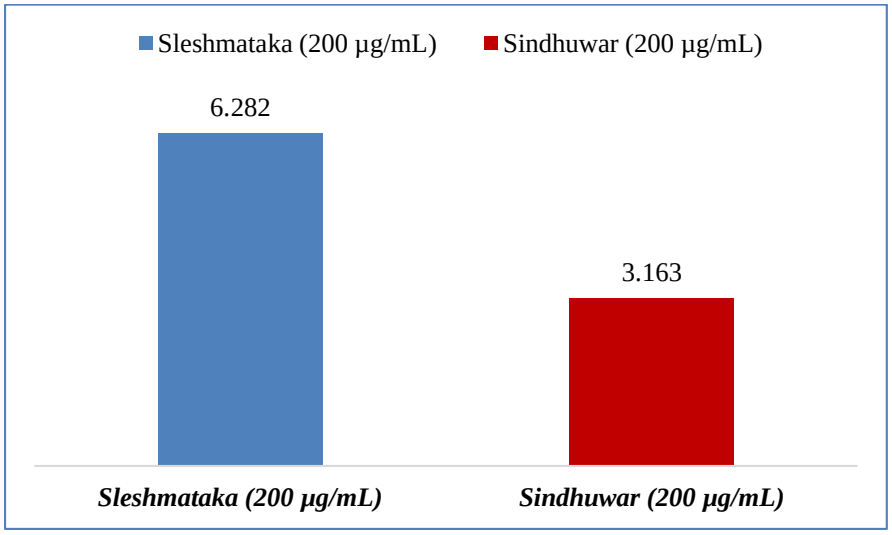
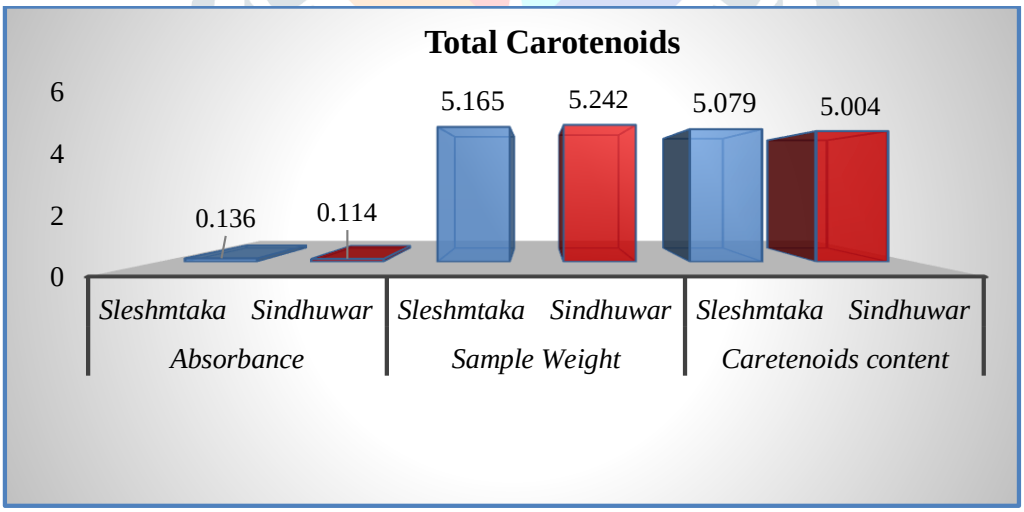


TABLE 4 - Results showing Total Carotenoids –

S. no	Test Sample	Absorbance	sample weight (gm)	Volume (ml)	Carotenoids content (µg/g)
1	***Sleshmataka***	0.136	5.165	50	5.079
2	***Sindhuwar***	0.114	5.242	50	5.004



STATISTICAL ANALYSIS-

A descriptive analysis was performed to describe the entire results within each kind of parameters. The difference among sets was compared by **unpaired t-test.** Unpaired t-test was carried out for comparison between two groups. The level of significance was set at P<0.005 for all analysis. The result on applying unpaired t-test is significant as P-Value was less than 0.005. Hence, it can be concluded that, there is significant difference between two groups (*sleshmataka and sindhuwar*). Statistical analysis was performed using SPSS v.20 software

DISCUSSION –

Inflammation is a vital physiological response of human immune system against foreign organism, including human pathogens, dust particles and viruses, hence helping in body's defense mechanism and healing process. Pain, heat, redness, swelling, and loss of function are the hallmarks of inflammation, which is characterised by dilated blood vessels that increase blood flow and by expanded intracellular spaces that allow leukocytes, protein, and fluids to move into the inflamed areas. To comprehend the function of inflammatory chemical mediators, this is crucial. These mediators arise from cells like neutrophils, platelets, mast cells, and monocytes/macrophages or are discharged as plasma proteins. They are set off by chemical or allergic irritants, trauma, and infections. These mediators—which are referred to as pro-inflammatory basic factors—determine the intensity of inflammation based on the length of the damage. These chemicals may improve vascular permeability, encourage neutrophil chemotaxis, stimulate smooth muscle contraction, boost direct enzymatic activity, cause discomfort, and/or mediate oxidative damage by binding to particular target receptors on the cells. Nitric oxide, prostaglandins, leukotrienes, vasoactive amines (histamine, serotonin), and cytokines are a few examples of chemical mediators. Although some of the released cytokines (IL-3, 4-, 5-, 6-, 10-, and 13) operate as a protective anti-inflammatory mediator within the cells. However, the state of chronic inflammation is a major factor for the progression of various chronic disease/disorder, including diabetes, cancer, cardiovascular disease, eye disorders, arthritis, obesity, autoimmune diseases, inflammatory bowel disease etc. Hence, it implies that substance having antioxidant and anti-inflammatory property will lead to better immunity and overall health of the individual. The present study was, therefore, aimed at investigating the in vitro antioxidant and anti-inflammatory activity of *sindhuwar and sleshmataka* with a view to justify the traditional use of these plant species in the treatment.

Effect of Protein Denaturation – Protein denaturation is a process in which proteins lose their tertiary structure and auxillary structure by application of outside push or compounds, such as solid corrosive or base, a concentrated inorganic salt, an organic solvent or heat. Most organic proteins lose their natural capacities when denatured. Denaturation of proteins is well documented cause of inflammation and leads to various inflammatory diseases including arthritis, stroke, cancer etc. The Tissue injuries happening in the human body during their lifetime can be referrable to denaturation of protein constituents of cells or of intercellular substances. Hence, the ability of a substance to inhibit the denaturation of the protein signifies apparent potential for anti-inflammatory activity. In this assay Bovine serum albumin was taken and incubated and heated on water bath. The optical density of the solution was measured at 660nm and percentage inhibition of precipitation/denaturation of Albumin protein was calculated.

The Potency of inhibition of denaturation by bovine albumin proteins of ethanolic extract of *sindhuwar* at the concentration of 5 % was 35.4%, 10% was 44%, 15% was 50%, and at **20% was 67%** & of *sleshmataka* at the concentration of 5% was 39.2%, 10% was 47.4%, 15% was 54.10% and at **20% was 76.10%**. On statistical comparison in between two drugs, Unpaired t-test was carried out for comparison between two groups and the results shows that P-Value was less than 0.05. Hence, it can be conclude that, there is significant difference

between two drugs (*Sleshmtaka* and *Sindhuwar*).Based on results, the inhibition rate of bovine albumin denaturation of ethanolic extract of *sleshmataka* found higher then ethanolic extract of *sindhuwar*.

Determination of Total Phenolic Content- Polyphenols are common compounds synthesized only by plants, with chemical features related to phenolic substances with reported bioactivities to balance oxidative and inflammatory push, to modify macronutrient digestion and to exert prebiotic- like effects on gut microbiota. The evaluation of the Total Phenolic Content (TPC) in each sample was carried out according to the procedure described by Sánchez-Rangel et al. (2013), Hernández-Carranza et al. (2019) and Kim et al. (2019), using FolinCiocalteu's reagent and UV-visible spectrophotometry. The absorbance was measured at 765 nm.The results were expressed as mg Total Phenols Gallic Acid Equivalent.A calibration curve for GA was prepared and R^2 was found to be 1 and y=0.132X -0.036. After solving the equation, the phenolic content of ethanolic extract of *sindhuwar* was **2.06mg/GAE** and that of ethanolic extract of *sleshmataka* was **5.538 mg/GAE**.On statistical comparison in between two drugs, Unpaired t-test was carried out for comparison between two groups and the results shows that P-Value was less than 0.05. Hence, it can be conclude that, there is significant difference between two drugs (*Sleshmtaka* and *Sindhuwar*).The result shows that ethanolic extract of *sleshmataka* has better phenolic content then ethanolic extract of *sindhuwar*.

Total Flavonoid Content (TFC) – The TFC was evaluated according to Hernández-Carranza et al. (2016, 2019). The absorbance was measured at 490 nm. Finally, a quercetin standard curve was performed. The result was expressed quercetin equivalent per 100 g.A calibration curve for quercetin was prepared and R^2 was found to be 0.997 and y=0.092X +0.034. After solving the equation, the flavonoid content of ethanolic extract of *sindhuwar* was 3.163QE/g and that of ethanolic extract of*sleshmataka* was 6.282 QE/g.On statistical comparison in between two drugs, Unpaired t-test was carried out for comparison between two groups and the results shows that P-Value was less than 0.05. Hence, it can be conclude that, there is significant difference between two drugs (*Sleshmtaka* and *Sindhuwar*). The result shows that ethanolic extract of *sleshmataka* has better flavonoid content then ethanolic extract of *sindhuwar*.

Total Carotenoid content- Carotenoid extraction was carried out according to Xavier et al. (2012).Absorbance was measured at λ 470 nm. Each extract was dissolved in n-hexane pro analysis. Total carotenoid content expressed as carotene equivalent per 100 g of ethanolic extract. Carotenoid Content of ethanolic extract of *sindhuwar* was **5.004** $\boldsymbol{\mu g/g}$ and *sleshmataka* is **5.079** $\boldsymbol{\mu g/g}$**.**On statistical comparison in between two drugs, Unpaired t-test was carried out for comparison between two groups and the results shows that P-Value was less than 0.05. Hence, it can be conclude that, there is significant difference between two drugs (*Sleshmtaka* and *Sindhuwar*).The results shows that ethanolic extract of *sleshmataka* has better Carotenoid content then ethanolic extract of *sindhuwar*.

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