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ORIGINAL ARTICLE **OPEN ACCESS**

Evaluation of Total Phenolic, Flavonoid, *In vitro* Antioxidant Potential and Anti-Inflammatory Activity of Successive Extracts of *Swertia Alata*.

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ABSTRACT
To perform phytochemical screening and to evaluate the total phenolic content, total flavanoidal content, invitro antioxidant and anti-inflammatory activities of various extracts of aerial parts of Swertia alata. In the present study all the extracts of aerial parts of S. alata was obtained by hot extraction method and subjected to preliminary phytochemical screening. The total phenolic content (TPC) and total flavanoidal content (TFC) was evaluated using gallic acid and quercetin calibration curve. The invitro antioxidant activity was evaluated as free radical- scavenging capacity (RSC). The RSC was assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) cation radical scavenging assay of successive extracts at different concentrations. Human RBC (HRBC) stabilization method and protein denaturation method were utilized to evaluate the invitro antiinflammatory activity of successive extracts at different concentration. Phytochemical analysis of all the extracts revealed the presence of major classes of phytochemicals such as glycosides, steroids, tannins etc. Both the found to contain the total phenolic content ranged from 13.58 to 150 mg/g gallic acid equivalents/g of dry extract and total flavonoid concentrations varied from 5.46 to 946.16 mg/g, Quercetin equivalents/g of dry extract. Among the extracts tested, ethanolic extracts of S. alata showed promising radical scavenging and anti-inflammatory activity. Based on the result of this pilot study, it can be concluded that S. alata is a good source of natural antioxidants which can be used to prevent progression of many chronic diseases. Further detailed phytochemical studies are needed to identify the chemical compounds responsible for exhibiting potent anti-inflammatory activity.
Key words: *Swertia alata, total phenolic content, total flavanoidal content, antioxidant, antiinflammatory*

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INTRODUCTION

Free radicals are chemical species which are highly reactive; possessing unpaired electrons in their outermost shell, which causes free radical damage (1). They are generated as by-products during normal cellular metabolism (2) .The free radicals can also be generated in biological systems in the form of reactive oxygen species (ROS), such as superoxide anion radicals (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), the singlet oxygen (O^2) and nitric acid (NO^{3-}) radicals (3).

The potentially reactive derivatives of oxygen are known as ROS which induces oxidative damage to various biomolecules including cell membrane disintegration, proteins, lipids, lipoproteins and deoxyribose nucleic acid (DNA) and initiate or propagate the development of many chronic and degenerative diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases, inflammatory diseases and also in the ageing process (4-6).

Plants have long been a source of exogenous (i.e., dietary) antioxidants. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of these have excellent antioxidant potential (7). Antioxidants are vital substances because they can protect the body from the damage caused by free radicals. Antioxidants exert their effect by scavenging the free radicals (i.e. reactive oxygen species (ROS) or reactive nitrogen species) universally present in biological systems (8). They delay or inhibit the oxidation of biomolecules by inhibiting the initiation or propagation of oxidative chain reactions and which can thus prevent or repair damage done to the body's cells by ROS (1).

Inflammation is one common and major cause of sufferings now and every time past. Inflammatory diseases including rheumatic diseases are a major cause of morbidity of the world (9).Inflammation is considered as a primary physiologic defence mechanism that help body to protect itself against infection, burns, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses (10).The currently available drugs like narcotics (e.g. opoids), non steroidal anti-inflammatory drugs (NSAIDS) e.g (Salicylates) and corticosteroids (e.g. hydrocortisone) are not useful in all cases of inflammatory disorders, because of their side effects and potency (11). These drugs act by inhibiting the function of prostaglandin. Prostaglandin is an autocoid that is released extracellularly and initiate pain. Anti-inflammatory agents block this autocoid synthesis by either inhibiting COX enzyme or protecting lysosomal membrane from breakdown (12). Signs of inflammation are swollen joints, joint pain, stiffness, and loss of joint functions. As a result, a search for other alternatives seems necessary and beneficial. The study of botanicals which are used traditionally to cure inflammation is still fruitful and logical research strategy in the source of new anti-inflammatory drugs (13).

Therapeutic potential of plants is mainly due to the presence of bioactive compounds. Plant constituents are responsible for both free radicals scavenging and anti-inflammatory activity. Secondary metabolites are responsible for biological activities of plants including terpenoids (14), phenolic compounds (flavonoids, phenolic acids, quinones, coumarins, lignans, stilbenes, tannins), and nitrogen compounds (alkaloids, amines, and betalains) and carotenoids (15). The rich wealth of plant kingdom can represent a novel source of newer compounds with significant antioxidant and anti-inflammatory activities.

Swertia alata Royle (Gentianaceae) is an annual herb commonly grows in west and north west himalayas particularly in kashmir to kumaon, mussorrie, dehradun and nanital region (16).An infusion of plant was known for its tonic, febrifuge and laxative properties. The plant contains three xanthones i.e swertiaperennine, swertianin and decussating with properties of curing malaria (17). It also contains oleanolic acid, ursolic acid swertiamarin, amarogentin and mangiferin which shows good antioxidant activity (18). The literature survey showed scanty information available on this plant and thus prompted us to analyze the common ayurvedic plant. The present study involves determination of phenolics by folin cioculteu method, flavanoids by aluminium chloride method, antioxidant activity by DPPH and ABTS assay and anti-inflammatory activity by membrane stabilization and albumin denaturation.

MATERIAL AND METHODS

Plant material and extraction

The dried plant material was supplied by an Ayurvedic Pharmaceutical Company, New Delhi and identified by Dr. H.B Singh, NISCAIR (National Institute of Science communication and Information Resources) Pusa Gate, New Delhi. The voucher specimen (NISCAIR/RHMD/2013/2185/191) of the test drug has been deposited in the herbarium of NISCAIR for future reference. The fresh aerial parts were dried under the shade and powdered in a mixer. The coarse powdered material (500g) of *S. alata* was extracted successively by petroleum ether, chloroform, ethanol and water. Each time before extraction with next solvents, the coarse powder material was dried in hot air oven below 50ºC. The extracts were evaporated to dryness under reduced pressure with a rotary evaporator (Heidolph) at a temperature of 40ºC. All the dried extracts were kept in tightly packed container under refrigeration until used for the biological testing.

Drugs and chemicals

The chemical used were Folin ciocalteau (FC) reagent, Aluminium chloride, Ascorbic acid, Diclofenac sodium, gallic acid and quercetin was purchased from sigma Aldrich. All other chemicals and reagents used were of highest analytical grade.

Phytochemical screening of extracts

The freshly prepared crude extracts of aerial parts of *S. alata* were subjected to qualitative phytochemical tests to check the presence of various classes of active chemical constituents such as tannins, saponins, glycosides, flavonoids, alkaloids, terpenes, steroids, etc. using standard procedure. (19)

Determination of total phenolic contents in the plant extracts

The concentration of phenolics in plant extracts was determined with the folin ciocalteau reagent using the method of spanos and wrolstad (1990) as modified by Lister and Wilson (2001) (20, 21). All the extract in the concentration of 1 mg/mL was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of solution of extracts, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL 7.5% NaHCO3. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 mL 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45ºC for 45 min. The absorbance was determined using spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of

absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/mL) from the calibration line; then the results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

Determination of flavonoid concentrations in the plant extracts

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (22). The sample contained 1 mL of methanolic solution of the extracts in the concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of QU/g of extract).

***In-vitro* Anti-oxidant activity**

DPPH radical scavenging activity

The ability of the plant extract to scavenge 1, 1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method (23). The stock solution of extracts were prepared in ethanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 20, 40, 60,80 and 100 µg/mL. Diluted solutions (1 mL each) were mixed with 3 ml of ethanolic solution of DPPH (DPPH, 0.004%). After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm using UV-Visible Spectrophotometer. Initially, absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured as control. Ascorbic acid was used as standard. The experiment was carried out in triplicate. Percentage inhibition was calculated using equation (1), whilst IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\text{\% inhibition} = \frac{\text{(Absorbance of control- absorbance of sample)}}{\text{Absorbance of control}} \times 100 \quad \text{equation (1)}$$

ABTS radical scavenging assay

For ABTS cation radical scavenging assay, the procedure followed the method with some slight modifications (24, 25). The stock solutions included 7mM $ABTS^+$ solution and 2.4mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at room temperature in the dark. The solution was then diluted by mixing 1ml $ABTS^+$. Solution with 60 ml methanol to obtain an absorbance of 0.706±0.001 units at734nm using the spectrophotometer. Fresh $ABTS^+$ solution was then prepared for each assay. Plant extracts/standard (1ml) of different concentration (20, 40, 60, 80,100 and 200 µg /mL) was allowed to react with 1ml of the $ABTS^+$ solution and the absorbance was taken at 734nm after 7min using the spectrophotometer. The $ABTS^+$ scavenging capacity of the extract was compared with that of Trolox and percentage inhibition calculated as:

ABTS radical scavenging activity (%) = $Abs_{control} – Abs_{sample}/Abs_{control} \times 100$ Where,

$Abs_{control}$ was the absorbance of ABTS radical + methanol, and

Abs_{sample} was the absorbance of ABTS radical + sample extract/standard.

Anti-inflammatory activity

HRBC membrane stabilization method

The HRBC membrane stabilization has been used as a method to study the anti-inflammatory activity (26). The blood was collected from median cubital vein of healthy volunteers and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% Sodium citrate, 0.05% citric acid and 0.42% Sodium chloride in water). The blood was centrifuged at 3000 rpm and packed cells were washed with isosaline (0.85% Sodium chloride, pH 7.2) and a 10% v/v suspension was made with isosaline. The assay mixture contained the drug (at various concentrations), 1ml phosphate buffer (0.15M, pH7.4), 2 mL of hyposaline (0.36%) and 0.5ml of HRBC suspension. Diclofenac sodium, a powerful non steroidal anti-inflammatory drug was used as a standard drug. Instead of hyposaline 2mL of distilled water was used as control. All the assay mixtures were incubated at 37ºC for 30 min and centrifuged. The haemoglobin content in the supernatant solution was estimated using spectrophotometer at 560nm.The percentage

haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%.The percentage of HRBC membrane stabilization or protection was calculated by using the formula,

Percentage protection = 100- (OD of drug treated sample/OD of control) ×100

Inhibition of albumin denaturation

The anti-inflammatory activity of all the extracts were studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al (27) and Sakat et al (28) followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 º C for 20 min, after cooling the samples the turbidity was measured at 660nm. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated by equation 1.

Data analysis

All experiments measurements were carried out in triplicate and are expressed as mean ± SD. The inhibitory concentration 50% (IC50) was calculated by plotting the data in the graph as concentration versus percentage inhibition using graph pad prism software, version 5.

RESULTS AND DISCUSSIONS

Extraction yield

The successive solvent extraction was done and extractive yield of Petroleum ether (PESA), Chloroform (CHSA), Ethanol (ETSA) and Aqueous (AQSA) extracts were calculated and given in Table 2. The yield of extraction by various solvents are in the following order PESA<CHSA<ETSA<AQSA. This shows that the extraction yield increases with increasing polarity of the solvent used in extraction. Compounds other than phenolics may have been extracted and contribute to higher yield. This may be attributable to the higher solubility of protein and carbohydrates in water not in organic solvent.

Preliminary phytochemical analysis

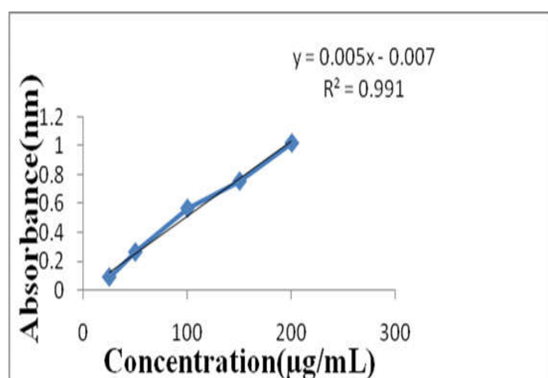
The various extracts of *S. alata* were tested for different phytoconstituents using standard procedures and results are given in Table 1. As given in Table 1 ETSA extract showed the presence of major classes of secondary metabolites such as phenolic, iridoids, tannins, carbohydrates, steroidal terpenes. AQSA extract showed absence of steroidal terpenes and iridoids while their presence was revealed in PESA extract.

Table 1: Qualitative Phytochemical investigation

Test	PESA	CHSA	ETSA	AQSA
Alkaloids	-	-	-	-
Flavanoids	-	-	+	+
Tannins	-	-	+	+
Saponins	-	-	-	+
Glycosides	-	-	-	+
Steroids	-	+	+	+
Steroidal terpenes	+	-	+	-
Phenolic	-	+	+	+
Gums and mucilage	-	-	-	+
Carbohydrates	-	-	+	+
Test for iridoids	-	-	+	-

Total phenolic content (TPC)

The total phenolic contents in PESA, CHSA, ETSA and AQSA of *S. alata* were examined using the folin-ciocalteu's reagent are xpressed in terms of gallic acid equivalent (the standard curve equation: y=0.005x-0.007, r^2 = 0.991).The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (Table 2).The TPCs were calculated using the above linear regression equation based on the calibration curve of gallic acid where y is absorbance and x is amount of gallic acid in µg(Figure 1). The total phenolic contents in the examined extracts ranged from 13.58 to 150 mg GA/g. It was found that the TPC of the extracts decreases with increase in polarity of solvent except for ethanol as solvent (Table 2). This may be because of content of more nonpolar compounds such as carbohydrates and glycosides in aqueous extracts than in other extracts. It may also be caused by the formation of a phenolic complex possessing higher molecular weight that is soluble in ethanol but insoluble in the aqueous extract (29). Based on the results of TPC, the best extracting solvent was ethanol.



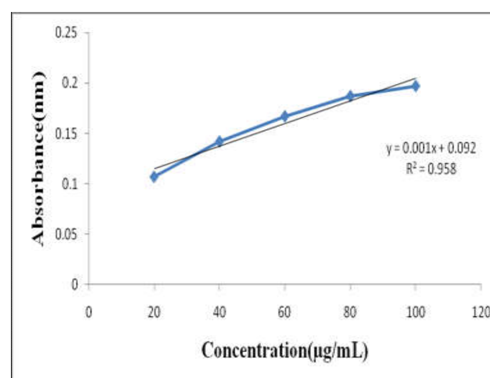


Figure 1: standard curve of gallic acid For TPC

Figure 2: standard curve of quercetin for TFC

Total flavanoidal content (TFC)

The concentration of flavanoids in various plant extracts of the species *S.alata* was determined using spectrophotometric method with aluminium chloride. The content of flavanoids was expressed in terms of Quercetin equivalent (the standard curve equation: y=0.001x+0.092, r^2=0.958), mg of Quercetin/g of extract (Table 2) where y is absorbance and x is amount of quercetin in µg (Figure 2).

The concentration of flavanoids in plant extracts from *S. alata* ranged from 5-946 mg/g. Chloroform and ethanol extracts contains the highest flavanoids concentration. The lowest flavanoidal concentration was measured in aqueous and petroleum ether. It was found that the effect of solvents on TFC is similar to that on TPC. The result is similar with the Chen et al. who reported the estimation of total phenolics and flavanoids in alcoholic solvents in the same genius (30).

Table 2: Result for Total Phenolic and flavanoidal content of extracts

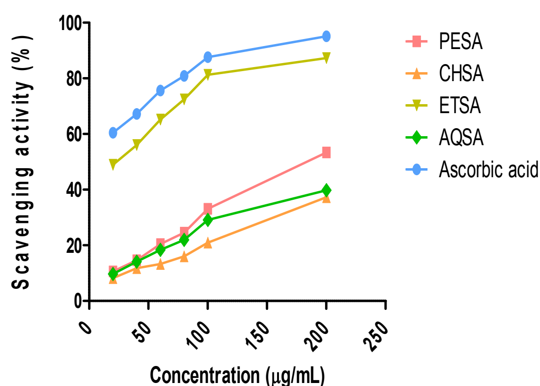
Extract	Yield (%) (w/w)	mg of GA/g of extract	mg of Quercetin/g of extract
PESA	1.968%,	13.58±0.4	110.16±0.65
CHSA	1.613%,	49.04±0.79	946.16±0.83
ETSA	7.963%	150±0.85	744.8±0.4
AQSA	9.012%.	91.84±0.57	5.46±0.60

values are expressed as mean±SD, n=3

In-vitro Anti oxidant Activities

DPPH free radical scavenging activity

The antioxidant activity of different plant extracts from *S.alata* was determined using an ethanolic solution of DPPH reagent. DPPH is very stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless-/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band (31). Fig 3 shows the DPPH scavenging activity in a concentration dependant manner. ETSA showed the strongest scavenging activity with an IC50 value of 25.91±0.46µg/mL which is comparable with the ascorbic acid (24.46±0.67 µg/mL), IC50 value of PESA (182.27±1.30 µg/ml), AQSA (250.61±1.20 µg/ml) and CHSA (281.60± 3.05 µg/ml) extract exhibited considerable antioxidant power. Arshya Hashim et al. reported a similar trend of DPPH radical scavenging activity of *Phyllanthus virgatus* extract (32).



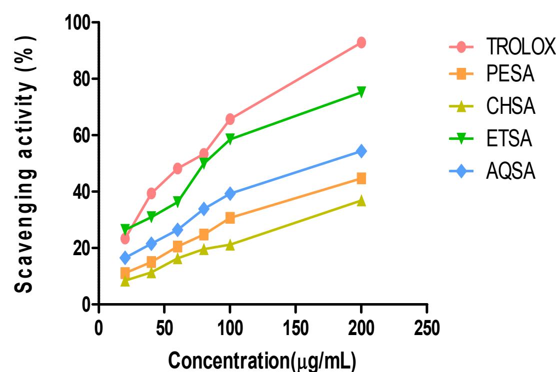


Figure 3: DPPH Scavenging activity **Figure 4: ABTS Scavenging activity**

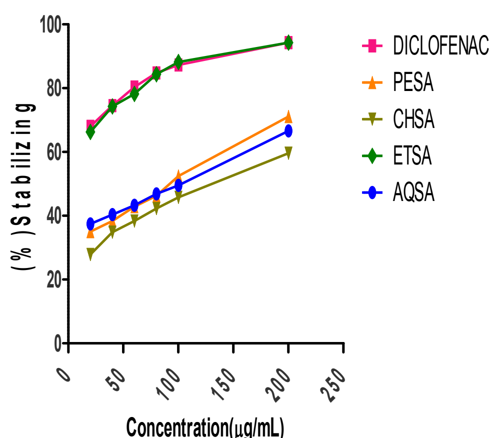
BTS assay

ABTS is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and Trolox (a water-soluble α-tocopherol analogue) (33). The ABTS scavenging activity of all the extracts of *S. alata* were detected and compared with Trolox. The results are expressed as percentage inhibition (% inhibition) at various concentration (20- 200 μg/mL) of PESA, CHSA, ETSA and AQSA extracts as well as standard Trolox (20 -200 μg/mL) were calculated and plotted in Fig 4 using Graph Pad Prism. The IC50 values are calculated from graph and were found to be 73.02μg/mL (Trolox), 220.03μg/mL (PESA), 282.27μg/mL (CHSA), 96.88μg/mL (ETSA) and 169.05μg/mL (AQSA) Extract. The IC50 of PESA, CHSA, AQSA are significantly lower than IC50 of trolox ($P<0.05$) is considered significant) while the IC50 of ETSA is comparable with IC50 of trolox. The results are expressed as % inhibition and are shown in figure 4. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and generally correlated with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom (34).

***Invitro* Anti-inflammatory Activities**

HRBC membrane stabilization

The erythrocyte membrane resembles to lysosomal membrane and as such the erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Stabilization of lysosomal is important in limiting the inflammatory response (35).The present studies on *S.alata* extracts for the stabilizing activity could lead to an increase in the surface area to volume ratio of the cells which may be brought about by an expansion of membrane or shrinkage of the cell and an interaction with membrane proteins according to chopade et al (36). It is therefore expected that phytochemicals present and their synergistic action as in extracts with membrane –stabilizing properties. This fact provides an evidence for membrane stabilization as an additional mechanism of its anti-inflammatory effect. The extracts were initially subjected to erythrocyte (RBC) membrane stabilization induced haemolysis by hypotonic solution and its effectiveness was dose dependent. All extracts may possibly inhibit the release of lysosomal content of neutrophillis at the site of inflammation. It was observed that ETSA extract showed significant activity when compared to the standard diclofenac sodium. The inhibitory effects of different concentrations of extracts on HRBC membrane stabilization are given in figure 5. The IC50 of PESA, CHSA, AQSA are significantly lower than IC50 of Diclofenac ($P<0.05$) and are considered significant while the IC50 of ETAH is comparable to IC50 of diclofenac and considered non significant. The IC50 values are given in Table 3. The activity of the extracts were concentration dependant, with the increasing concentration the activity is also increased. These results may be attributes due to the presence of phenolic content and good anti-inflammatory properties.



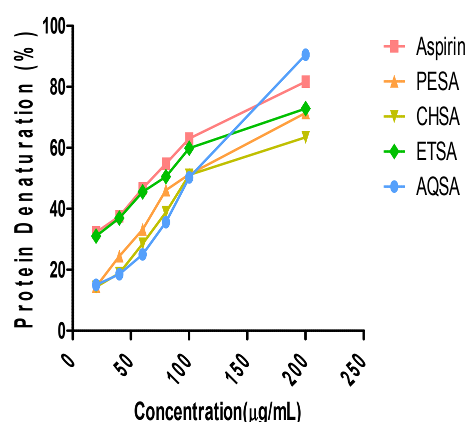


Figure 5: Percentage stabilization **Figure 6: Percentage denaturation**

Albumin denaturation

Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti inflammation activity, ability of extract protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation as per Table 3 and figure 6. Maximum inhibition was observed from ETSA followed by AQSA, CHSA and PESA at the concentration of 200 µg/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition 81.73% at the concentration of 200µg/mL. The IC50 value of ETSA extract (74.70µg/mL) is considered non significant as compared to standard (Aspirin) value 68.05±1.75 µg/mL as per Table 3.

The data in Figure 6 showed that all the extract showed significant inhibition in dose dependant manner. The IC50 of PESA extract ($p<0.01$), CHSA ($p<0.001$) and AQSA ($p<0.0001$) are considered significant as compare to IC50 of Asprin ($P<0.05$).

Table 3: Fifty percent inhibitory concentration (IC50) of *S. alata* on Membrane Stabilization and Protein denaturation

Extracts of *S. alata*	IC50 (µg/mL)	
	Membrane stabilazation	**Protein denaturation**
Diclofenac/ Aspirin	10.05±1.08	68.05±1.75
PESA	94.79±2.41***	93.86±1.4*
CHSA	135.02±5.07***	102.01±1.27**
ETSA	13.45±1.14^{ns}	74.70±0.86^{ns}
AQSA	99.63±1.51***	133.613±2.39***

*Each value represents the mean ± SD, n=3, and results were analyzed by one way ANOVA followed by Dunnet test. ***p<0.0001, considered extremely significant, **p<0.001, *p<0.01, p<0.05 are considered significant; ns p>0.05, non significant.*

CONCLUSION

In the present study, results indicate that the ethanolic extract of *S. alata* possess anti-oxidant and anti-inflammatory properties. In general, extraction yield increased with increasing polarity of organic solvent that facilitates the extraction of all compounds that were soluble in both water and organic solvents. The TPC of extracts were consistent with the TFC, antioxidant and anti-inflammatory activity of extracts. Identification, isolation and purification of each bioactive compound of this plant may in future yield a natural antioxidant and anti-inflammatory agent for the treatment of various diseases such as cancer, inflammation, neurological disorder and aging.

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