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Phytochemical profiling and anti-inflammatory potential of *Ficus natalensis* subsp. *leprieurii* (Miq.) C.C. Berg – an *in vitro*, *in vivo* and *in silico* study

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Abstract: Inflammation is immune response of body against foreign toxic substances. Chronic inflammation is associated with diabetes, heart diseases, arthritis and cancer. The present study aims to investigate anti-inflammatory potential of plant *Ficus natalensis* subspecies leprieurii. Various extracts of leaves of *F. natalensis* were investigated for bioactive phytochemicals and screened using *in vitro* activities of anti-inflammatory. *In vivo* study of anti-inflammatory potential was also conducted by using method of carrageenan induced paw edema. This plant showed potential *in vitro* protein denaturation assay of anti-inflammatory activity. *In vivo* assay revealed that n-hexane extract exhibited the maximum percentage inhibition of paw volume (83.49%) as compared to standard drug used. GC-MS analysis of n-hexane extract exhibited tentative identification of bioactive phytochemicals which were further evaluated for their activity by using molecular docking. The results suggested that gamma tocopherol has the highest binding affinity with BSA. The results concluded that *F. natalensis* has medicinal importance and promising anti-inflammatory potential.

Keywords: *Ficus natalensis,* small molecules, anti-inflammatory, GC-MS, molecular docking.

INTRODUCTION

Inflammation is normal response of body to injury or infection characterized by pain, redness, swelling and change in physiological functions. Body's defense mechanism against viral, bacteriological or chemical agents is termed as inflammation due to which certain chemical mediators are produced from damaged tissue (Galeotti and Bayry, 2020). Chronic inflammation results into other diseases like atherosclerosis, ischemic heart disease, rheumatoid arthritis, hay fever, inflammatory bowel syndrome, asthma, tuberculosis, nephritis and celiac disease etc. (Ginwala *et al*., 2019).

Secondary metabolites present in medicinal plants are used in the treatment of ailments. Compounds extracted from plants have therapeutic and biological roles against harmful infectious organisms. These compounds are major source of development of new drugs. Population living in villages and rural areas in developing countries use folk herbal medicine. One of the limitations of allopathic medicines is the strong belief of people on herbal medicine (Aziz *et al*., 2018). Tomatine, a glycoalkaloids, found in wild tomato fruit has reported pharmacological effects on humans including cholesterol lowering, immunomodulator and cardiotonic. Secondary metabolites like tannins, trypsin inhibitors and lectins found in wild bean specie of Phaseolus vulgaris have benefit for health in therapy for diabetes, cardiac disease and cancer. Digitoxin and digoxin are cardiac glycosides found in plant digitalis purpurea used for treatment of cardiac ailment like congestive heart failure. Similarly, sterols, sitosterol, stigmaterol and campesterol are reported to lower serum cholesterol. Morphine, codeine, thebaine, papaverine and noscapine are examples of opioids alkaloids found poppy plants. Opioids alkaloids are used as sedative, analgesics and cough suppressants (Schmidt *et al*., 2008).

It was reported that alkaloids, terpenes, saponins, tannins, flavonoids, steroids and glycosides found in abundance in *F. natalensis* plant. These secondary metabolites has powerful zones of inhibition against a few chosen human infections with antibacterial activity (Sheyin *et al*., 2018). *F. natalensis* plant material is a potent source of antibacterial and antioxidant compounds. More impressive results came from antibacterial activity than from antifungal activity (Ajaib *et al*., 2016). A chemical composition study was performed essential oil extracted from leaves of *F. natalensis.* (E) phytol (37.6%) and 6,10,14 trimethyl-2-pentadecanone (24.9%) were reported in essential oil extracted from plant *F. natalensis*. Derivatives of (E) phytol, acetate and epoxide phytol found in *F. natalensis* were reported to active against mycobacterium. Phytol has anticancer properties. This high concentration of phytol in *F. natalensis* suggested this plant as potential source of phytochemicals (Sonibare *et al*., 2009).

In recent years, allopathic medicines produced harmful effects that limit the beneficial use of the drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) are

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commonly used allopathic medication in the treatment of inflammation. Prolong use of NSAIDs results into adverse effect including stomach ulcers, adrenal suppression (Sharma *et al*., 2021).Compounds isolated from plants are preferred due to their low adverse effects, low cost, low morbid and safer in use. Herbal medications reduce the severity of illness and extend the life expectancy (Farrukh *et al*., 2022). Pharmacological and therapeutic activities are responsible for presence of different photochemical present in the plants. This suggested to discover/isolate the compounds having anti-inflammatory potential to achieve the better pharmacological response with minimum adverse effects (Banu *et al*., 2020).

There are hundreds of phytochemicals compounds in the *F. natalensis* plant which are not characterized. In this study, different extracts of plant material were analyzed for *in-vitro* and *in-vivo* anti-inflammatory potential. The aim of this study is to identify the compounds from complex phytochemical profile of *F. natalensis* by using extraction and GC-MS technique. Computer aided molecular docking was performed on compounds identified from GC-MS data to predict bioactive compounds in *F. natalensis* to understand their anti-inflammatory roles using mechanism of denaturation of protein.

MATERIALS AND METHODS

Materials

Analytical and HPLC grade chemicals and reagents including methanol, ethanol, chloroform and n-hexane were used. Gallic acid, aluminum chloride, quercetin, sodium carbonate, hydrate sodium acetate, Folin-Ciocalteu (FC) reagent, sodium phosphate dibasic dihydrate ($Na_2HPO_4.7H_2O$) (Merck, Germany), sodium phosphate monobasic monohydrate ($Na_2HPO_4.H_2O$) (Merck, Germany), concentrated hydrochloric acid (HCl), bovine serum albumin (BSA) (Sigma) and carrageenan (Biochem Ltd, China) were used.

Collection, identification, pulverization of plant material

The plant material was collected from Bagh-e-Jinnah and Botanic Garden of Government College University (GCU), Mall Road, Lahore, Pakistan. Part used (leaves) of the plant was subjected to authentication from the Department of Botany, Government College University (GCU), Lahore, Pakistan. Voucher number GC.BOT-HERB-4002A were allocated to leaves of *F. natalensis* by GCU herbarium, Lahore. Leaves of *F. natalensis* subsp. *leprieurii* (Miq.) C.C. Berg were clean with water and allowed to dry under shade. Dried plant material was crushed and pulverized into powder. It was stored in an airtight container at room temperature.

Preparation of plant extracts

Extraction of plant material was performed by method of sequential hot extraction in soxhlet extractor (Pyrex, USA) using solvents of different polarities i.e. n-hexane, chloroform and methanol. The powdered leaves were also subjected to ethanol and water solvents by cold extraction method (Islam *et al*. 2015). Extracts were concentrated using rotary vacuum evaporator (HEI-VAP series, Heidolph, Germany). Percentage yield (%) of all extracts was calculated individually by their respective final weight relative to the weight of dried powder taken initially. The extracts were stored at 25^{o}C for analysis.

Phytochemical analysis by qualitative methods

Bioactive chemical constitutes in the leaves extracts of *F. natalensis* were screened and identification of secondary metabolites by chemical analysis were performed utilizing the standard protocols described by Harborne (1998) and Sofowora (1993). The standard tests for qualitative analysis of primary metabolites carbohydrates, proteins, fats and secondary metabolites like anthraquinones, alkaloids, flavonoids, phenols, glycosides, steroids, resins, saponins and tannins, were performed on extracts of *F. natalensis* (Nortjie *et al*., 2022).

Quantitative estimation of total phenolic, flavoniod and protein contents

Total phenolic contents (TPC)

Total phenolic contents of leaves extracts of *F. natalensis* were quantified by using spectrophotometric method (Saidan *et al*., 2015, Slinkard and Singleton, 1977). Gallic acid was used as standard reference to determine the total phenolic content in plant extracts of *F. natalensis*. Briefly, 1mg/mL of different extracts were prepared by using methanol as solvent. Gallic acid was also prepared in concentration of 1mg/mL in methanol (stock solution). Different concentration of standard solution ranging from 10 to 400µg/mL (10, 20, 40, 80, 100, 200, 400) were prepared in methanol solvent. Then, 50µL each of extract and standard solution concentrations were taken and 200µL of folin ciocalteu (FC) reagent in each test tube. Add 4mL of distilled water in each reaction tube. The tubes were incubated at 25^{o}C for 10 minutes. After incubation period, add 750µL of sodium carbonate (20 % w/v) in each test tube. The reaction test tubes were again incubated at 25^{o}C for 60 minutes in dark. After incubation, the absorbance of each standard and extract reaction test tubes was measured against blank at wavelength 765nm by using spectrophotometer. In blank, in place of sample, 50µL methanol was used. TPC was expressed as milligram (mg) equivalents of standard gallic acid per gram of dry matter (mg GA/g). Polyphenols concentration in different extracts of *F. natalensis* was determined from standard curve of concentration of gallic acid ranging from 10 to 400 µg/mL.

Total flavonoid contents (TFC)

Total flavonoid contents (TFC) were quantified in different extract sample of plant leaves by following

method of Chandra *et al*. (2014). Standard reference quercetin was used for determination of TFC. Stock solution of quercetin was prepared by dissolving 5mg in 1mL of methanol. Different concentration dilution of quercetin ranging from 10µg/mL to 120µg/mL were prepared. Each extract was prepared in concentration of 1mg/mL using methanol as solvent. 300µL of each extract solution was mixed with 60µL of 10% aluminum chloride solution. Then, add 60µL of 1M sodium acetate solution along with 900µL of methanol. 1.7mL of distilled water was used to make volume upto 3mL. After mixing, all test tube were incubated at 25°C for 60 minutes. Absorbance of reaction solution against blank sample was measured at wavelength 510nm by using spectrophotometer. Total flavonoid content in each extract was determined from the curve of standard quercetin and expressed as equivalent of mg quercetin (QE)/g.

Estimation of total protein contents

Total protein contents in crude extracts of *F. natalensis* were estimated by following protocol described by Saidan (Saidan *et al*., 2015) with slight modifications. Analytical reagent use in this analysis was freshly prepared. Solution-A was prepared by mixing 0.1M sodium hydroxide with 2% w/v of sodium carbonate. Solution-B was prepared by mixing 1.56 % w/v copper sulfate with 1% potassium sodium tartrate. The analytical reagent was prepared by mixing 100mL of Solution-A with 2mL of Solution-B. Labeled this analytical reagent as Solution-C. Bovine serum albumin (BSA) was used to prepare standard solution in concentration of 1mg/mL. 10 mg of all extracts of *F. natalensis* was mixed with 10mL of distilled water. Each test and standard solution were vortexed. Then, test solution was centrifuged at 2700rpm for 10 minutes. 0.1mL of supernatant was collected from each sample. The separated supernatant solution was made to 1mL with distilled water. 3mL of Solution-C was added in test tubes containing supernatant solution. The reaction test tubes were incubated for 10 minutes at 25°C. After incubation, 200µL of folin-ciocalteu's (FC) reagent was added. All reaction test tubes were incubated again for 30 minutes at 25°C. Absorbance of reaction test samples and standard against blank. was measured at wavelength 600nm using spectrophotometer. Blank sample containing all reagents except crude extract. Different concentration of standard BSA ranging from 25µg/mL to 1000µg/mL were prepared to obtain calibration curve.

Anti-inflammatory activity

Bovine serum albumin (BSA) protein inhibition activity

Leaves extracts of *F. natalensis* were investigated for the anti-inflammatory potential using *in-vitro* model of BSA protein inhibition method. The assay was carried out by the previous research with minor modification (Saleem *et al*., 2020). BSA solution was prepared with distilled water in 5 % w/v concentration. 0.45mL of BSA solution was mixed with distilled water to prepare the control solution. Stock solution of different extracts in concentration of 1mg/mL was prepared. Add 0.45mL of BSA in 0.05mL of each extract to prepare test solution of extracts. Product control solution was prepared by adding 0.45mL of distilled water in 0.05mL of different extract solution. Diclofenac sodium is used as standard drug and its solution was prepared by addition of 0.05mL of 1% diclofenac sodium in 0.45mL of BSA. All solutions were incubated for 20 minutes at 37°C in an oven and then for 3 minutes at 60°C in a water bath. All test samples were cooled at room temperature and temperature was maintained at 37°C for analysis. Take 2.5mL of phosphate buffer having pH 7.4 in all reaction test tubes. Absorbance of standard, tests and control solutions was measured at wavelength of 660 nm by using Ultra Violet (UV) Visible Spectrophotometer. Percentage inhibition was calculated by the formula mentioned below (Saleem *et al*., 2020). This was determined by comparing test solutions to standard diclofenac sodium drug.

$$\%\ inhibition = 100 - \frac{absorbance\ of\ test\ solution - absorbance\ of\ product\ solution}{absorbance\ of\ control} \times 100$$

Egg albumin denaturation inhibition activity

Egg albumin denaturation method was used with slight modification to investigate plant extracts of *F. natalensis* for *in-vitro* anti-inflammatory activity (Chandra *et al*., 2012). Albumin of fresh hen's egg was used in this method. Different extract solution of plant *F. natalensis* were prepared by dissolving 1mg of crude extracts in 1mL of distilled water. Standard drug solution of diclofenac sodium was also prepared in similar manner (1mg/mL). 0.2mL of egg albumin was added in 5mL reaction mixture containing 2.8mL of phosphate buffer saline having pH 7.4 and 2mL of crude extracts solution. Standard drug solution was also prepared by replacing extract solution with 2mL of stock diclofenac sodium solution. Product control solutions were prepared by adding 0.2mL of distilled water instead of egg albumin. Control solution was prepared with 2mL of distilled water. Incubate all test solutions at 37°C for 15 minutes and then placed for 5 minutes at 70°Cin water bath. Cooled at room temperature (25°C). Absorbance of all reaction mixtures was measured at wavelength of 660nm by using spectrophotometer. Percentage inhibition was calculated by the following formula (Chandra *et al*., 2012).

$$\%\ inhibition = 100 - \frac{absorbance\ of\ test\ ssample - absorbance\ of\ product\ control\ solution}{absorbance\ of\ control} \times 100$$

In vitro membrane stabilization activity on human red blood cells

RBCs were assayed in-vitro with slight modification using membrane stability to screen plant extracts of *F. natalensis* in order to determine maximum percentage inhibition (Ahsan *et al*., 2021). In this assay, red blood cells of those healthy volunteers were selected who were not taking NSAIDs for last 14 days. Blood of health volunteers was collected in vacutainer from Mayo

Hospital, Lahore. Stock solution of plant extracts and standard drug, in concentration of 1mg/mL, was prepared. 10% v/v suspension of collected blood of healthy volunteers was suspended with sterile normal saline solution (0.9% NaCl). This suspension was centrifuged for 10 minutes at 3000rpm. Supernatant was collected and discarded. Repeat this procedure 2 -3 times until the collected supernatant was clear. Add 10mL normal saline solution in the obtained pellet. Test solution contained 0.5mL of extract solution, 2mL of hypotonic solution (0.36% w/v NaCl), 0.5mL 10% v/v RBCs and 1mL 0.15M sodium phosphate buffer (pH 7.2). Standard drug solution contained 0.5mL diclofenac sodium in place of test extract sample. Control test sample was prepared by replacing 2mL hypotonic solution with distilled water. All assay samples were incubated for 30 minutes at 37^{o}C followed by centrifugation at 3000rpm for 10 minutes. Upper layer was collected. Absorbance was measured at wavelength 560nm by using spectrophotometer. Normal saline solution was used as a blank. % RBCs membrane stabilization was measured by following equation (Ahsan *et al*., 2021):

$$\%\ \text{RBCs membrane stabilizatio} = 100 - \frac{absorbance\ of\ tests\ sample}{absorbance\ of\ control} \times 100$$

Carragreenan induced edema in paw (In vivo anti-inflammatory activity)

Carrageenan induced edema in animal model was used to investigate *in-vivo* anti-inflammatory potential of extract of plant *F. natalensis* (Saleem *et al*., 2021). Male albino mice that weighed between 30 to 40g were selected. They were kept in a regular laboratory setting at a temperature of 25°C. To assess anti-inflammatory properties, albino mice were grouped into five, each with six (06) mice (n=6): control, disease, standard drug, extract group received 100mg of n-hexane extract and extract group received 200mg of n-hexane extract of *F. natalensis*. Standard drug used was diclofenac sodium. The dose was determined for each mouse based on body weight prepared in normal saline. Following an hour of pre-treatment, each mice received an injection of 0.1mL of freshly made carrageenan. A dose of the extract, standard drug and carrageenan were injected into the mice's left paw at the sub-plantar location. After injecting carrageenan, diclofenac sodium and extract, paw size was measured using digital vernier caliper at time intervals of 0, 1, 2, 3, 6 and 12 hours. For the average paw swelling across all groups, the standard deviation was calculated, and one-way ANOVA was applied followed by Tukey's Post-hoc Test with p-value ($p<0.05$) was considered significant. The Punjab University Institutional Ethics Review Board approved the study for animal experiments and issued the voucher number D/382/FIMS.

Gas chromatography mass spectrophotometer (GC-MS) analysis of leaves extract of F. natalensis

GC-MS technique was used for analysis of crude extract of powdered leaves of *F. natalensis*. Length of capillary tube was 30m and diameter is 0.25 nm with film coating of 0.25µm. Carrier gas Helium at flow rate of 0.25mL/min was used. Sample was prepared by dissolving extract in methanol. The column pressure was 0.77 psi. The volume of the sample was 1µL. Temperature of oven was maintained at 100^{o}C for 5 minutes. Afterwards, temperature was increased gradually at rate 10^{o}C for 10 minutes till reach desired temperature of 200°C. At the end, temperature was elevated to 325°C for 30 minutes. Various peaks were obtained for different compounds evaluating extract of *F. natalensis* leaves. Compounds present in the extract were interpreted by comparing the peaks in the database of National Institute of Standards and Technology NIST20 having minimum quality factor of 90.

In silico studies

Molecular docking was performed on the GC-MS selected 11 compounds using computer-aided drug designing software, Schrodinger Maestro Suite. Ligand molecules were selected based on their pharmacological activities and significant peak regions. The docking technique is useful to understand the mechanism of interaction of protein receptors with ligands. Structures of the protein, BSA were downloaded from website of protein data bank (PDB: 4JK4), while ligands structures were prepared with Schrodinger Maestro Suite software. Three-dimensional conformation of protein BSA was formed with Protein Preparation Wizard in software. Ligands and water molecules were removed from protein structure. Ligand structures were positioned around enzyme's active site. Using Glide software in Maestro, molecular docking was performed (Imtiaz *et al*., 2022). Prime MM-GBSA analysis was done using OPLS 2005 force field on the top 3 metabolites that showed maximum docking score using Schrodinger Maestro Suite.

STATISTICAL ANALYSIS

For each of aforementioned assays and activities, three biological replicate extracts were examined. Results were expressed after subtracting the negative control or blank sample reading (without extracts). Mean and standard deviation (S.D) of the mean were calculated for these results using computer based software Microsoft Excel 2019. One-way analysis of variance (ANOVA) followed by Post-hoc Tukey's Test was applied with p-value ($p<0.05$) was considered to be significant.

RESULTS

Phytochemical analysis

Qualitative analysis

After subjecting powdered leaves of *F. natalensis* to extraction, obtained extracts were dried and weighed accurately. The percentage yield of methanolic, ethanolic, chloroform, n-hexane and aqueous extracts were 16.25%, 22.82%, 2.61%, 8.53% and 8.11% respectively.

Table 1: Phytochemical constituents' analysis of extracts of *F. natalensis*

S No.	Metabolite	n-Hexane extract	Chloroform extract	Ethanolic extract	Methanolic extract	Aqueous extract
Primary Metabolites						
1.	Carbohydrates	+	+	+++	+	+++
2.	Lipids	+++	++	++	-	++
3.	Proteins	+	+	+	+	++
Secondary Metabolites						
1.	Alkaloid	++	-	++	+	+
2.	Anthraquinone	+	+	+	+	+
3.	Flavonoid	++	++	++	+	-
4.	Glycoside	-	-	++	++	+
5.	Phenol	+	++	++	++	+
6.	Resin	-	-	-	-	+
7.	Saponin	++	+	+	+	++
8.	Steroid	++	-	+	+	+
9.	Tannin	+	-	++	++	++

+ indicates slight presence, ++ indicates moderate presence, +++ indicates strong presence, - indicates absence.

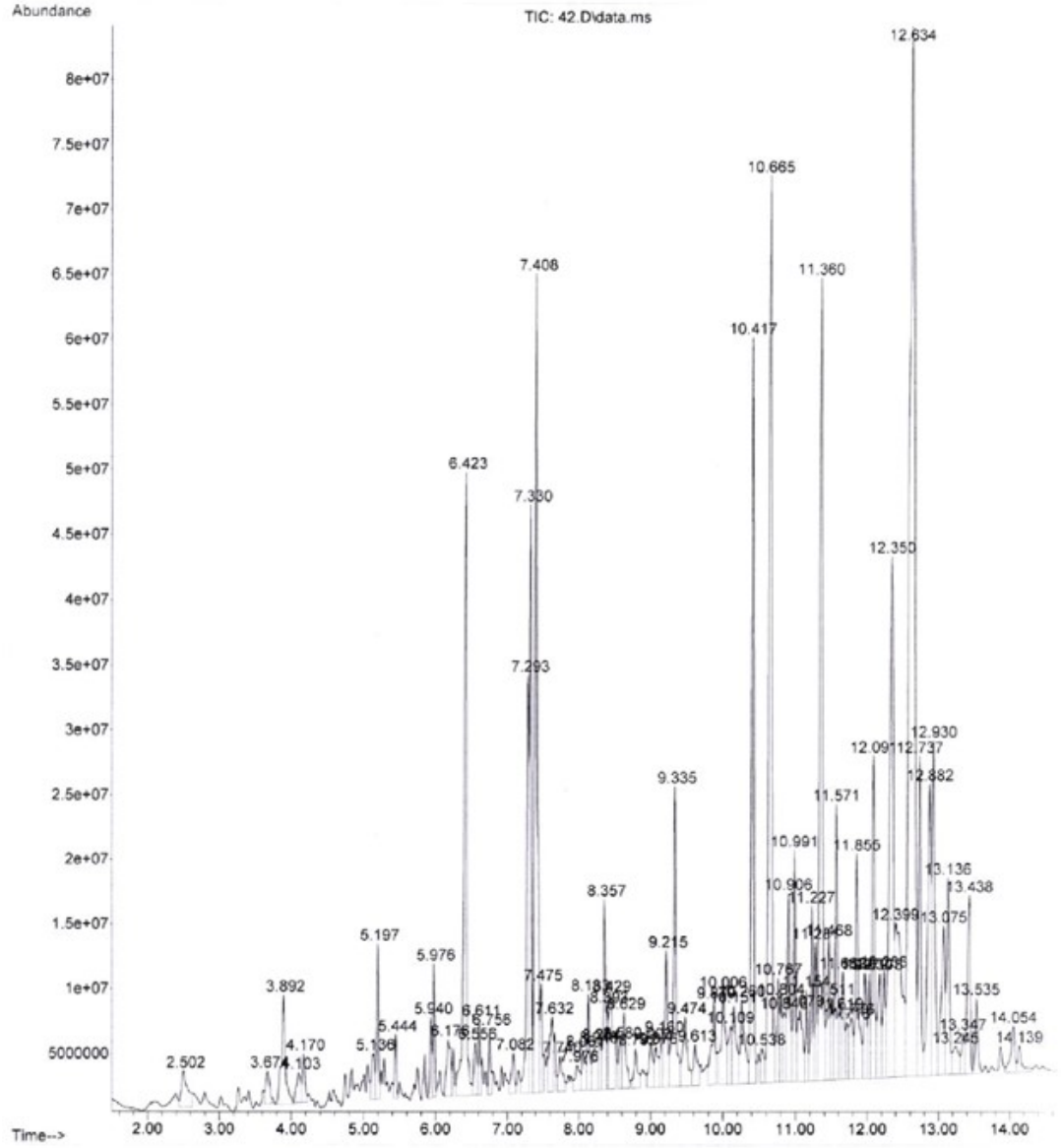

Fig. 1: GC-MS chromatogram of n-hexane extract of *F. natalensis*

Table 2: Total protein, phenolic, flavonoids contents of extracts of *F. natalensis*

Extracts	Total Protein	Total Polyphenols	Total Flavonoids
n-Hexane	11.93±0.35	28.76±0.22	107.00±0.84
Chloroform	24.56±0.48	48.58±0.76	99.34±0.70
Ethanol	37.44±0.74	36.39±0.31	111.25±0.75
Methanol	29.18±0.88	56.97±0.65	36.82±0.34
Aqueous	45.39±1.26	27.24±0.11	12.57±0.14

Table 3: Inhibition of protein denaturation by BSA

Sample	Absorbance	% Denaturation inhibition of BSA
n-Hexane	0.048±0.003*	42.17
Chloroform	0.079±0.007	4.82
Ethanol	0.071±0.006	14.46
Methanol	0.065±0.004*	21.69
Aqueous	0.061±0.004*	26.51
Standard Drug (Diclofenac Sodium)	0.042±0.001*	49.40

(n=3), *p<0.05, Significant relative to control reading (Control = 0.083)

Table 4: Inhibition of protein denaturation by egg albumin

Sample	Absorbance	% inhibition of denaturation of egg albumin
n-Hexane	0.054±0.003*	68.42
Chloroform	0.129±0.008*	24.56
Ethanol	0.165±0.006	3.51
Methanol	0.156±0.006	8.77
Aqueous	0.138±0.005*	19.30
Standard Drug (Diclofenac Sodium)	0.021±0.001*	87.72

(n=3), *p<0.05, Significant relative to control reading (Control = 0.171)

Table 5: Red blood cell membrane stabilization activity

Sample	Absorbance	Prevention of lysis of RBC (%)
n-Hexane	0.235±0.056*	45.98
Chloroform	0.385±0.074	11.49
Ethanol	0.325±0.023*	25.29
Methanol	0.367±0.082	15.63
Aqueous	0.314±0.045	27.82
Standard Drug (Diclofenac Sodium)	0.174±0.021*	60.00

(n=3), *p<0.05, Significant relative to control reading (Control = 0.435)

Table 6: Comparison of size of paw against carrageenan induced inflammation and treated with standard drug and n-hexane extract

		Carrageenan-induced paw edema			
Time (hr)	Control group (mm)	Disease group (mm)	Standard Drug (5 mg/kg) (mm)	Group treated with extract (100mg/kg) (mm)	Group treated with extract (200mg/kg) (mm)
0	2.40 ± 0.34	2.18 ± 0.98*	1.59 ± 0.33*	2.33 ± 0.68*	1.98 ± 0.44*
1		2.98 ± 0.68*	1.76 ± 0.23*	3.09 ± 0.70*	2.61 ± 0.91
2		3.35 ± 0.43*	2.08 ± 0.50*	3.74 ± 0.89*	3.47 ± 0.28*
3		3.98 ± 0.57	2.45 ± 0.40*	3.78 ± 0.55	3.66 ± 0.86*
6		4.15 ± 0.76*	2.77 ± 0.39*	3.12 ± 0.98	3.00 ± 0.78
12		4.59 ± 0.36	2.32 ± 0.68*	2.82 ± 0.32*	2.29 ± 0.30*

mm: size of the paw
*p<0.05, Significant relative to control reading
Results were represented as mean ±S.D (n = 6), analyzed by one way ANOVA and Post-hoc Tukey's Test.

Table 7: Important biological active profile of n-hexane extract of *F. natalensis* by GC-MS analysis

S No.	Compound Name	Formula	Molecular weight	Area %	Retention Time	Category	Pharmacological actions
1.	Hexahydrofarnesyl acetone (2 pentadecanone, 6, 10, 14 tri methyl)	$C_{18}H_{36}O$	268.48	0.49	5.976	Sesquiterpene Essential Oil	antibacterial, anti-nociceptive and anti-inflammation activities (Avoseh *et al*., 2021)
2.	Squalene	$(C_5H_8)6$ $[C_{30}H_{50}]$	410.73	3.71	10.417	Triterpene isoprenoid	Anti-aging, Anti-inflammatory. Squalene is investigated as an adjunctive cancer therapy (Lozano-Grande *et al*., 2019)
3.	Delta Tocopherol	$C_{27}H_{46}O_2$	402.65	1.23	10.906	Methylated phenol	Anticancer, Antioxidant (Abraham *et al*., 2019)
4.	Beta Tocopherol	$C_{28}H_{48}O_2$	416.7	0.88	11.227	Methylated phenol	antioxidant, anti-inflammatory potential (Abraham *et al*., 2019)
5.	Gamma Tocopherol	$C_{28}H_{48}O_2$	416.7	0.61	11.281	Methylated phenol	Antidermatitic, anticancer, hepatoprotective, antioxidant, antispasmodic (Abraham *et al*., 2019)
6.	Campesterol	$C_{28}H_{48}O$	400.7	0.65	11.970	Phytosterol, steroids derivatives	Cholesterol-lowering properties, antidiabetic, anticancer, activities, anti-inflammatory effect (Mustafa *et al*., 2022)
7.	Gamma Sitosterol	$C_{29}H_{50}O$	414.7	4.74	12.350	phytosterols	antidiabetic, antihyperglycemic activity (Balamurugan *et al*., 2011)
8.	Beta Amyrone	$C_{30}H_{48}O$	424.70	2.34	12.399	Triterpenoids	anti-inflammatory, antifungal Activity (de Almeida *et al*., 2015)
9.	Lupeol	$C_{30}H_{50}O$	426	1.77	12.737	pentacyclic triterpenoid	Anti-inflammatory activity, Anti-cancer (Saleem, 2009)
10.	Beta Amyrin	$C_{30}H_{50}O$	426.7	2.20	12.930	pentacyclic triterpenoid	anti-inflammatory, antinociceptive, gastroprotective and hepatoprotective properties (Nogueira *et al*., 2019)
11.	urs-12-en-24-oic acid 3-oxo- methyl ester	$C_{31}H_{48}O_3$	468.7	1.30	13.136	Triterpenoids	anti-inflammatory, anti-cancer agent (Oh *et al*., 2021)

Table 8: Molecular docking of important biological active profile of n-hexane extract

Compounds identified in GCMS	Docking score	Glide Score	Glide emodel
Gamma Tocopherol	-3.764	-3.764	-36.471
Beta Tocopherol	-3.379	-3.379	-38.773
Delta Tocopherol	-3.039	-3.039	-43.328
Squalene	-2.090	-2.090	-34.674

Table 9: Prime MM-GBA data of relative binding free energy (Kcal/mol)

Compounds identified in GCMS	MMGBSA-dG-binding energy	MMGBSA-dG-Bind coulomb	MMGBSA-dG-bind (NS)	MMGBSA-dG Bind (NS)-coulomb
Gamma Tocopherol	-56.16	-19.71	-61.22	-19.14
Beta Tocopherol	-50.69	-7.43	-89.38	-6.64
Delta Tocopherol	-68.51	-3.05	-87.17	-3.12
Squalene	-76.56	-9.55	-86.48	-9.63

"MMGBSA dG Bind = Complex − Receptor – Ligand. "MMGB

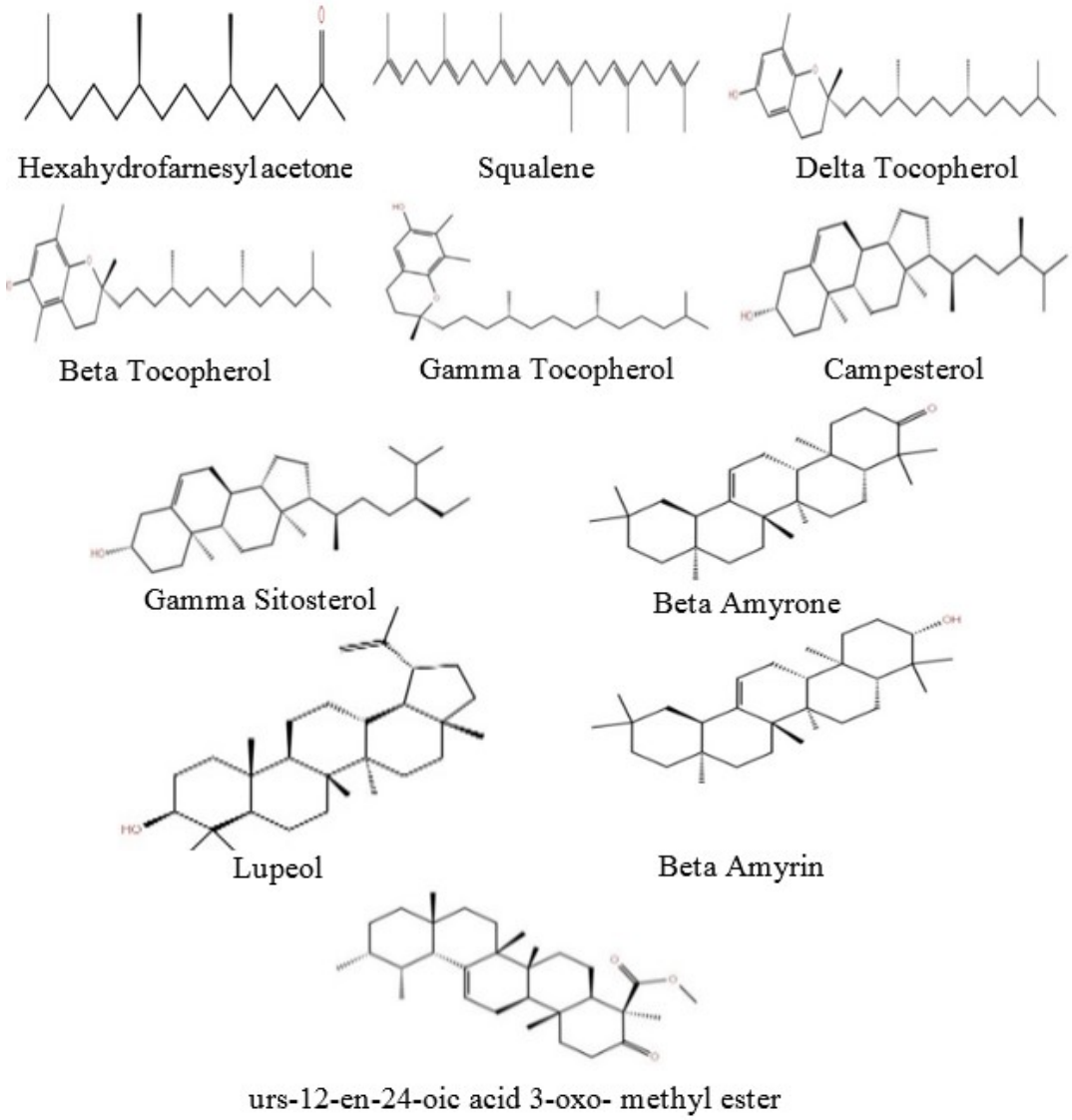

Fig. 1: Structures of ligands used for molecular docking studies

Preliminary phytochemical analysis was performed on leaves extracts of *F. natalensis*. Five extracts including methanolic, ethanolic, chloroform, n-hexane and aqueous of leaves of *F. natalensis* were evaluated in detail. Phytochemical screening of leaves extracts revealed presence of different primary and secondary metabolites. Carbohydrates were found in aqueous and ethanolic extracts of leaves and only minor found in other evaluated extracts. Lipids were not identified in methanolic extract, while identified in other extracts. Proteins were identified in aqueous extract with minor in other extracts. Secondary metabolites including anthraquinones, flavonoids, saponins and phenols were identified qualitatively in all tested extracts of *F. natalensis*. Alkaloids were identified in all extracts except chloroform extract. Steroids and Tannins were not found in chloroform extract. Resins were not identified in any of extracts of plant *F. natalensis* (table 1).

Quantification of total phenolic, flavonoids and protein contents

Aqueous extract of *F. natalensis* showed maximum concentration of protein with value of 45.39±1.26 (%) after evaluating with standard Bovine Serum Albumin (BSA). Maximum concentration of TPC was quantified in methanolic extract with value of 56.97±0.65 mg of gallic acid equivalent per gram of extract (mg GAE/g), and lowest concentration was observed in aqueous extract (27.24±0.11).

Highest concentration of TFC was found in the ethanolic extract 111.25±0.75mg QE/g extract), and lowest concentration was found in aqueous extract (12.57±0.14 mg QE/g extract). Total flavonoid content values for n-hexane and chloroform found to be substantially identical (107.00±0.84 and 99.34±0.70mg QE/g extract,

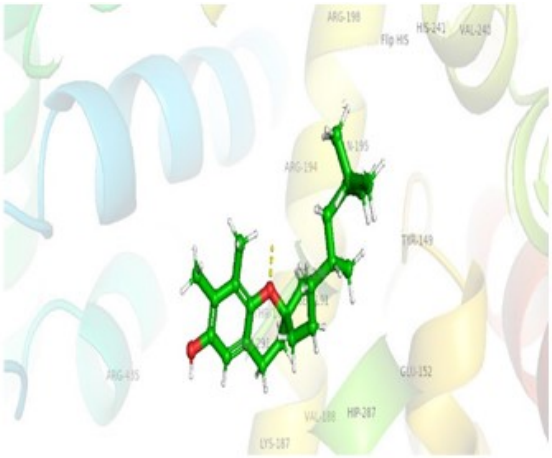

Fig. 3.1: Gamma tocopherol docked in binding site of protein.

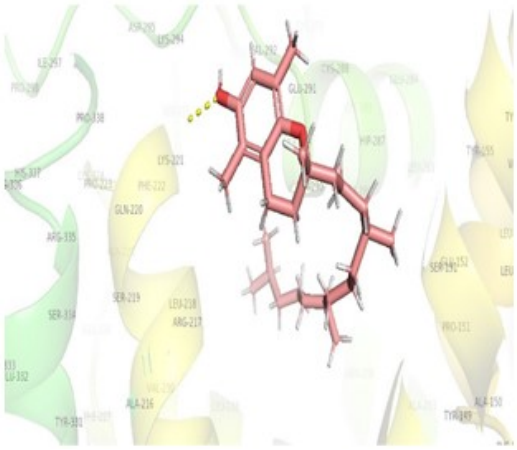

Fig. 3.2: β-tocopherol docked in binding site of protein.

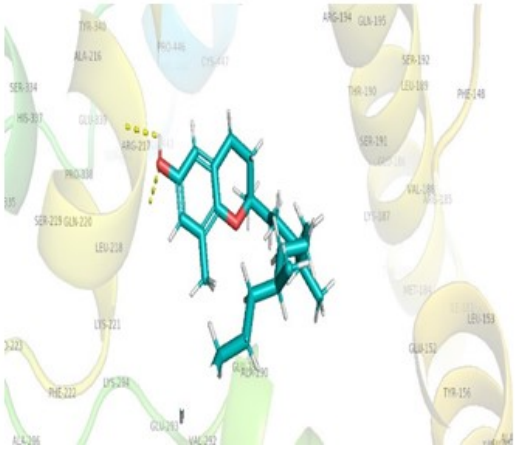

Fig. 3.3: Delta tocopherol docked in binding site of protein.

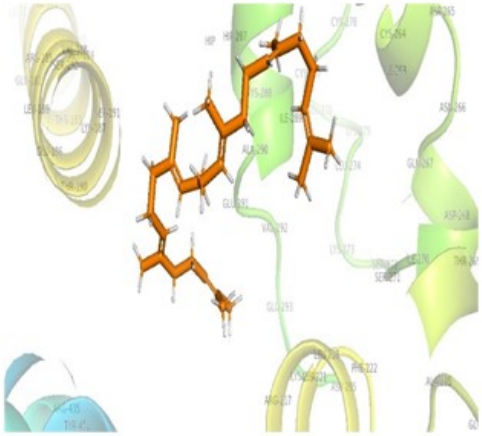

Fig. 3.4: Squalene docked with protein.

Fig. 2: Attachment of ligands with the binding site of protein (3.1) Binding of BSA protein with gamma tocopherol ligand 3D illustrated (3.2) Binding of BSA protein with beta tocopherol ligand 3D illustrated (3.3) Binding of BSA protein with delta tocopherol ligand 3D illustrated (3.4) Binding of BSA protein with squalene ligand 3D illustrated

respectively). These numbers forecast possible biological potential of the plant (table 2).

Anti-inflammatory biological activity

Measurement of inhibition of protein denaturation by BSA

As shown in table 3, crude extracts of plant *F. natalensis* indicated a significant inhibition of BSA protein denaturation. The highest inhibition was observed in n-Hexane extract (42.17%) followed by aqueous extract (26.51%).

Measurement of protein denaturation inhibition by egg albumin

Results of *in-vitro* egg albumin denaturation assay of all extracts of *F. natalensis* are summarized in table 4. N-Hexane extract had shown a greater potential for inhibition of protein denaturation (68.42%) and results were comparable with diclofenac sodium used as standard drug in treatment of inflammation (87.72%). Order of anti-inflammatory potential is n-hexane > chloroform > aqueous > methanol > ethanol.

Measurement of red blood cell membrane stabilization

Results on *in-vitro* anti-inflammatory activity by measuring red blood cell membrane stabilization are presented in table 5. Result showed n-hexane extract of *F. natalensis* protects human erythrocytes membrane from lysis. Crude n-hexane extract of *F. natalensis* leaves produced 45.98% protection against RBC hemolysis as compared with standard drug diclofenac sodium that produce 60 % protection. The prevention of hypotonicity induced membrane lysis of RBC was considered as potential activity of anti-inflammatory drugs.

Inhibition of carrageenan induce paw edema

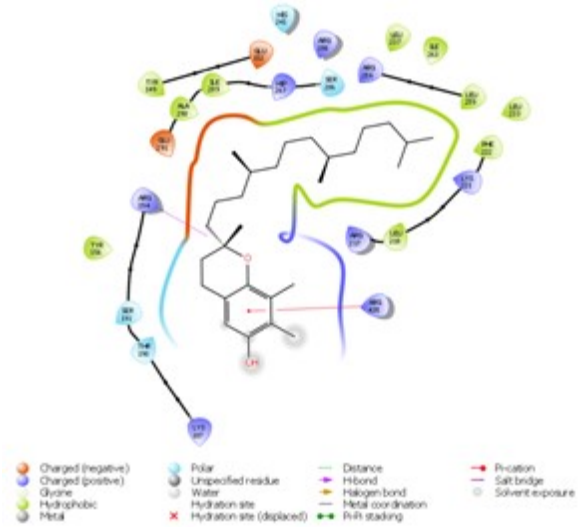

Fig. 4.1: Contact site of ligand gamma tocopherol with amino acid ARG 194 and ARG 435 with pi cation and nonpolar bond with other amino acids.

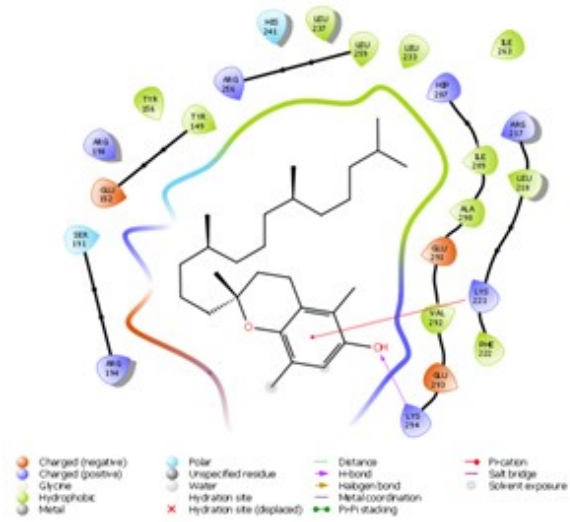

Fig. 4.2: Contact site of ligand beta tocopherol with amino acid LYS 221 and LYS 294 and nonpolar bond with other amino acids.

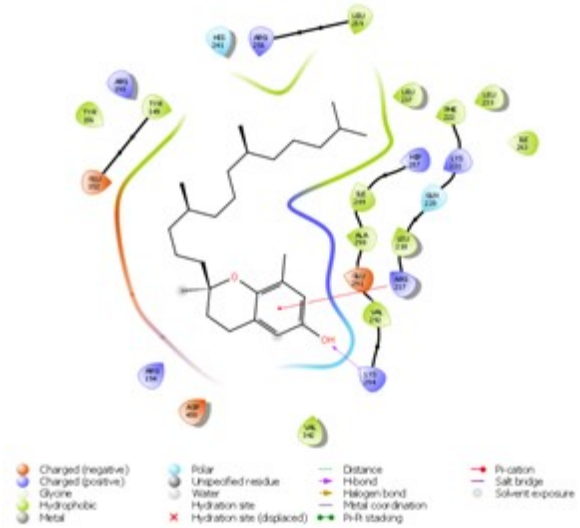

Fig. 4.3: Contact site of ligand delta tocopherol with amino acid LYS 221 and ARG 217 and nonpolar bond with other amino acids.

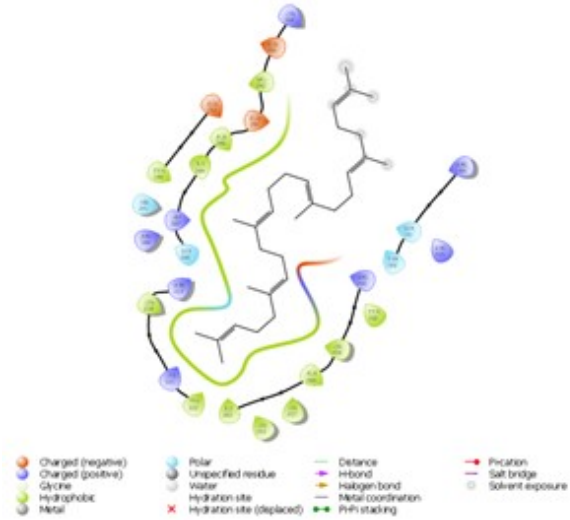

Fig. 4.4: Ligand squalene bonded by nonpolar with amino acids.

Fig. 3: Estimated binding geometry of gamma tocopherol, beta tocopherol and delta tocopherol with BSA amino acid residues. Polar bonds with amino acids are presented with arrows (4.1) Contact site of ligand gamma tocopherol with amino acid ARG 194 and ARG 435 with pi cation, and nonpolar bond with other amino acids (4.2) Contact site of ligand beta tocopherol with amino acid LYS 221 and LYS 294, and nonpolar bond with other amino acids (4.3) Contact site of ligand delta tocopherol with amino acid LYS 221 and ARG 217, and nonpolar bond with other amino acids (4.4) Ligand squalene bonded by nonpolar with amino acids

Anti-inflammatory potential was also assessed *in-vivo* by carrageenan-induced paw edema with n-hexane extract of *F. natalensis* leaves. N-hexane of leaves extract of *F. natalensis* exhibited the maximum percentage inhibition of paw volume (83.49±0.79%) at 100mg/kg dose and was comparatively higher than that of standard drug diclofenac sodium (69.87±0.53%). The width of the paws was reduced by n-hexane extract and diclofenac sodium, as shown by their size reduction of inflammation (table 6). The results showed a significant activity of the n-hexane extract treated group as compared with standard drug treating group. However, the reduction in the paw size of mice was slightly more prominent in the extract treated group instead of standard drug treated group

which might depend on route of injection or dose - dependent effect of n-hexane extract of *F. natalensis* leaves.

Identification of compounds by GC-MS analysis

GC-MS analysis of most potent crude extract of *F. natalensis* exhibiting anti-inflammatory potential was performed. This revealed a distinctive separating ability to authenticate, identify and determine compounds. N-hexane extract showed promising anti-inflammatory potential and was subjected to GC-MS analysis. Non-polar compounds are found in n-hexane extract of *F. natalensis*. National Institute of Standard and Technology (NIST) library was used to identify the compounds present in n-hexane extract (fig. 1). Various peaks obtained for different compounds were interpreted by comparing them to the database of NIST. GS-MS of n-hexane extract results identified 81 compounds. Compounds with more than 90% similarity index were selected. Name of compounds molecular formula, retention time (RT) in minutes and percentage area (% area), chemical class and pharmacological activity of compounds identified by GC-MS in the n-hexane extract are listed in table 7 and their structures are shown in fig. 2. Major compounds identified are hexahydrofarnesyl acetone, squalene, beta tocopherol, delta tocopherol, gamma tocopherol, campesterol, gamma sitosterol, beta amyrone, lupeol, beta amyrin, urs-12-en-24-oic acid 3-oxo-methyl ester having reported promising anti-inflammatory and other pharmacological activity.

In silico molecular docking

Eleven compounds identified from n-hexane extract by GC-MS were selected and docked against the BSA protein. Four docked compounds showed the highest binding energies (-2.090 to -3.764Kcal/mol) with arginine (ARG) active site. The escalated negative docking score (-3.764 Kcal/mol) represented the high binding affinity (-50.69 Kcal/mol) of gamma tocopherol with BSA protein. This receptor ligand complex described the biological activity of the compound. Beta tocopherol docking score was -3.379Kcal/mol with binding affinity -68.51 Kcal/mol. While squalene showed the lowest docking score of -2.090 Kcal/mol as compared to others.

The ligand gamma tocopherol was surrounded by amino acids ASP 236, VAL 234, LEV 233, VAL 240, LEV 237, LEV 259, SER 286, ILE 289, ALA 290, HIP 287, LEU 274, VAL 292, GLU 291, THR 291, LYS 187, TYP 156, SER 191, VAL 188, ARG 435, ALA 261, PHE 222, LYS 221 and LEU 218 through non-polar bonding.

Similarly, ligand beta tocopherol formed hydrogen bonding with LYS 294 amino acid of BSA protein. Ligand beta tocopherol was surrounded by amino acids ARG 194, SER 191, GLU 152, ARG 198, TYR 156, TYR 149, ARG 256, LEU 259, LEU 257, HIS 241, LEU 233, HIP 287, ILE 263, ARG 217, LEU 218, ILE 289, ALA 290, GLU 291, VAL 292, LYS 221, PHE 222 and GLU 293 through non-polar bonding.

Delta tocopherol ligand also formed hydrogen bonding with LYS 294 amino acid of BSA protein. Ligand was link by pi-cation with ARG 217 amino acid. Delta tocopherol was surrounded by amino acids LEU 259, ARG 256, HIS 241, ARG 198, TYR 156 TYR 149, GLU 152, ARG 194, ASP 450, LEU 237, PHE 222, LEU 233, HIP 287, LYS 221, ILE 289, ILE 263, GLN 220, LEU 218, ALA 290, VAL 292, GLU 291 and VAL 342 through non-polar bonding.

Ligand squalene surrounded with amino acids of protein (BSA) by non-polar bond. These amino acids included PHE 222, LYS 221, LEU 218, ARG 217, SER 286, HIP 287, ARG 198, HIS 241, TYR 149, ILE 289, ALA 290, GLU 152, GLU 291, VAL 292, GLU 293, LYS 294, ARG 194, SER 191, ARG 435, THR 190, ARG 256, TYR 156, ALA 260, LEU 259, ILE 263, LEU 233 and LEU 237.

According to Prime MM-GBSA method, relative binding free energy of each ligand was calculated. Energy of optimal free receptors, free ligand, complex ligand and receptor are calculated by the Prime MM-GBSA. Gamma tocopherol binding energy to BSA protein, was 56.16 Kcal/mol, while squalene showed the highest energy – 76.56 Kcal/mol. The relative binding energies of four molecules are described in table 9.

DISCUSSION

The metabolic activities typically result in formation of reactive oxygen species (ROS). Inflammation and tissue damage are caused by an excessive buildup of ROS, which negatively affects proteins, DNA, and fatty acids. Therefore, these ROS should be detoxified with antioxidants in order to improve the immune system (Magder, 2006). Plants contain biologically active substances called polyphenols and flavonoid molecules (Asghar *et al*., 2018, Saleem *et al*., 2018). Several benefits, including antibacterial, antidiabetic, antioxidant, antiviral, anti-inflammatory, anti-allergic and anticancer properties are associated with polyphenols and flavonoid molecules (Imtiaz *et al*., 2017, Yousuf *et al*., 2022). During literature survey and in the best of knowledge, the anti-inflammatory potential of the plant *F. natalensis* is not discussed before.

F. natalensis was subjected to extensive phytochemical screening using conventional laboratory testing in the current investigation. The extracts of *F. natalensis* contain moderate to high levels of secondary metabolites including phenols, flavonoids, alkaloids, glycosides, lipids, tannins, saponins and steroids after phytochemical screening. Phytochemical analysis of bark and leaves

extracts of plant *F. natalensis* was reported to contains alkaloids, terpenoids, tannins, flavonoids, anthraquionones, cardiac glycosides, saponins and reducing sugar (Lai *et al*., 2010). The previous studies did not indicate presence of saponins, steroids in the extracts of plant *F. natalensis.* These metabolites has been documented as anticancer, anti-aging, antiviral, antifungal, cardioprotective, contraceptive properties and antimicrobial properties (Elekofehinti *et al.,* 2021).

Alkaloids have properties of analgesic, anticancer and antimicrobial action. Flavonoids and tannins also reported as antibacterial and antioxidant agents (Lai *et al*., 2010). Saponins have potential as anti-inflammatory, antibacterial, anti-diabetic and anti-cancer agent (Urzúa *et al*., 2008). Therapeutic potential of *F. natalensis* extracts may be contributed by the presence of certain phytochemicals.

Inflammation may also be occurred because of denaturation of protein. Protein denaturation may contribute to the development of auto-antigens in several rheumatic disorders. Protein denaturation is known to be inhibited by anti-inflammatory medications. Due to their ability to prevent protein denaturation, nonsteroidal anti-inflammatory medications are the main pharmacological agents utilized for the management of inflammation and pain (Geenen *et al*., 2018). As a result, substances that can stop denaturation could be employed to create anti-inflammatory medications.

Different methods like bovine serum albumin, heat-induced denaturation of egg albumin and membrane stabilization of red blood cell were selected for assessment of *in-vitro* anti-inflammatory potential of plant *F. natalensis*. These are validated, dependable, and sensitive tests, frequently used methods for anti-inflammatory activity of pharmacophores. Decreased absorbance of tested extracts of *F. natalensis* compared to the reference drug suggested that the extracts had an anti-inflammatory effect by stabilizing proteins in a dose-dependent way. This is a new discovery for extract of *F. natalensis* leaves.

The phytochemicals discovered in *F. natalensis* contribute to this plant's anti-inflammatory properties. Quercetin and other phytochemicals have been demonstrated to stimulate the release of bifidobacterial anti-inflammatory agents that can reduce the formation of nitric oxide in macrophages that is caused by LPS (Kleemann *et al*., 2011).

Tentative identification of compounds in n-hexane extract of *F. natalensis* by GC-MS revealed presence of hexahydrofarnesyl acetone, squalene, beta tocopherol, delta tocopherol, gamma tocopherol, campesterol, gamma sitosterol, beta amyrone, lupeol, beta amyrin, urs-12-en-24-oic acid 3-oxo-methyl ester with antibacterial activity, anti-nociceptive, anti-aging, anti-inflammatory, antioxidant properties, hepatoprotective, anticancer, antispasmodic, antidiabetic, antihyperglycemic properties (table 7). The study could also be beneficial in the assessment of anti-rheumatoid potential of the extract by inhibiting the inflammation process (Saleem *et al*., 2021).

For a better understanding of molecular underpinnings of biological activity of the natural products, *in silico* studies were effectively used for theoretical prediction of ligand-target interaction. Additionally, it offers more information on the potential method of action and manner of binding of substances that are active against enzymes (Imtiaz *et al*., 2023). Eleven compounds from GC-MS profile of n-hexane extract were docked against BSA protein, in order to gain a better understanding of studied compounds' ability to inhibit enzyme and correlate results of experimental enzyme inhibition. Four compounds of n-hexane extract (Squalene, alpha, beta and gamma tocopherol) were docked against BSA. Conclusively, this confirms our finding of anti-inflammatory potential of extract of plant *F. natalensis* in terms of protein denaturation inhibition assays.

CONCLUSION

The findings of this investigation demonstrated that *F. natalensis* plant's leaves extract have strong anti-inflammatory action. N-hexane extract having terpenoids, essential oil, methylated phenol, phytosterol and other compounds, showed moderate to good anti-inflammatory potential. This is a groundbreaking discovery that the *F. natalensis* plant has compounds that can be used as an anti-inflammatory agent. *F. natalensis* could be a promising source for usage in natural products, medicines, and nutraceuticals due to its high potential for anti-inflammatory activity. A safe and effective anti-inflammatory medicine, as well as maybe an anti-arthritic drug, could be developed using extracts of *F. natalensis*. Further research design on isolation of compounds from extracts of *F. natalensis* and their structure elucidation is currently in progress.

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