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Research Article

IN SILICO DOCKING STUDIES AND *IN VITRO* XANTHINE OXIDASE INHIBITORY ACTIVITY OF COMMERCIALLY AVAILABLE TERPENOIDS

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Abstract

Objective Xanthine oxidase is a highly versatile enzyme that is widely distributed among different species. The hydroxylation of purines is catalysed by xanthine oxidase and especially the conversion of xanthine to uric acid. Xanthine oxidase inhibitors are much useful, since they possess lesser side effects compared to uricosuric and anti-inflammatory agents. The present study deals with *in silico* and *in vitro* xanthine oxidase inhibitory analysis of commercially available terpenoids (bisabolol, β-caryophyllene, limonene, and α- terpinene).

Methods Molecular docking studies were performed using AutoDock 4.2 and *in vitro* xanthine oxidase inhibitory activity was carried out using xanthine as the substrate. In addition, enzyme kinetics was performed using Lineweaver Burkplot analysis. Allopurinol, a known xanthine oxidase inhibitor was used as the standard.

Results The results revealed that bisabolol exhibited a lowest binding energy value of about -7.33 kcal/mol. All other compounds showed binding energy values ranging between -7.33 to -5.87 kcal/mol which was less than the standard (-4.78 kcal/mol). In the xanthine oxidase assay, IC_{50} value of bisabolol was found to be 34.70 µg/ml, whereas that of allopurinol was 8.48 µg/ml. All the remaining compounds exhibited IC_{50} values ranging between 34.70 to 68.45 µg/ml. In the enzyme kinetic studies, bisabolol, β-caryophyllene showed non competitive and Limonene, α- terpinene and allopurinol showed competitive type of enzyme inhibition.

Conclusion It can be concluded that terpenoids could be a promising remedy for the treatment of gout and related inflammatory disorders. Further *in vivo* studies are required to develop potential compounds with lesser side effects.

Keywords: Xanthine oxidase; Terpenoids; Binding energy; Enzyme kinetics; Gout

1. Introduction:

Drug discovery is a linear process that begins with a target and lead, which is followed by lead optimization and *in vitro* and *in vivo* screening to determine if such compounds satisfy the pre-formulated criteria for initiating clinical development. Most of the discoveries in the past were by either identifying the active ingredient present in traditional remedies or by accidental discovery[1]. Earlier, new drugs were developed by synthesizing compounds through a multi-step processes which was time consuming. This was followed by in *vivo* screening and further investigating their pharmacokinetic properties. But this lead to high failure rates due to poor pharmacokinetics, lack of efficacy, animal toxicity, adverse effects in humans and various miscellaneous factors[2].

Now-a-days applying computational methods for drug discovery and development are increasingly gaining in popularity, implementation and appreciation[3]. Since the process of drug discovery and development consumes very much time and resources there is an ever growing focus in the area of computational technique to streamline drug discovery, design, development and optimization. Most widely used techniques are ligand-based drug design (pharmacophore, a 3-D spatial arrangement of chemical features needed for biological activity), structure-based drug design (drug-target docking), and quantitative structure-activity relationships[4].

Among different computational methods, molecular docking programs, have become an integral part of the drug discovery program. The docking programs can be divided mainly into two categories, stochastic or random approaches[5]. Molecular Docking is a computational technique that predicts the

preferred orientation of one molecule to a second when bound to one another to form a stable complex which in turn predicts the strength of association or binding affinity between these molecules[6]. The various docking methods differ in the use of receptor representation, ligand treatment, scoring function and search algorithm. There are about 60 docking softwares and more than 30 scoring functions. Generally more than one search method and scoring function are provided so as to increase the accuracy of the simulations. Simulated annealing and genetic algorithm is the most commonly applied optimization method and more than 50% uses "force field" based scoring functions[7]. AutoDock 4.2 has been widely used for virtual screening, because of its enhanced docking speed. It is based on Lamarckian Genetic Algorithm (LGA) which is a hybrid genetic algorithm with local optimization that uses a parameterized free-energy scoring function to estimate the binding energy[8].

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are the interconvertible forms of the same gene product, xanthine oxidoreductase. This is a large homodimer protein with molecular weight of 290 kDa and has 2 flavin molecules (as FAD), 2 molybdenum atoms and 8 Fe atoms per enzyme unit. The enzyme is made up of around 1,330 aminoacids and the sequence is highly homologous among the mouse, rat and human enzymes with about 90% identity[9]. It is found inside the cells, where it performs its role in purine degradation. Uric acid is the final breakdown product of unwanted purines in humans because higher primates lack the enzyme uricase that, in other species, converts uric acid into allantoin. The biosynthesis of uric acid is catalyzed by the enzyme xanthine oxidase (XO) and/or its isoform, xanthine dehydrogenase[10].

Xanthine oxidase inhibitors (XOI) are much useful, since they possess lesser side effects compared to uricosuric and anti inflammatory agents[11]. Allopurinol is the only clinically available XOI, which also suffers from many side effects such as hyper sensitivity syndrome, Steven's Johnson syndrome and renal toxicity.

A wide variety of higher plants act as a major source for natural products used in pharmaceuticals, agrochemicals, flavor, fragrance ingredients and food additives[12]. Terpenoids encompasses a vast, diverse group of natural products which are found in various forms in most organisms where they fulfil a broad range of functions. These terpenoids also include industrially useful polymers (e.g., rubber and chicle) and also agrochemicals (e.g., pyrethrins and azadirachtin). A wide variety of medicinal plants are known to improve several medical conditions due to the presence of certain phtyochemicals. For example, the anti-malarial drug Artemisinin and the anticancer drug paclitaxel are two of a few terpenoids with established medicinal values. These are not only useful as herbal medicines but also in dietary use[13].

Thus, there is necessary to develop compounds with XOI activity with lesser side effects when compared to allopurinol. We thus began our work to look for *in silico* Docking studies and *in vitro* xanthine oxidase inhibitory activity of commercially available terpenoids.

2. Experimental details

2.1 Softwares required: Python 2.7 - language was downloaded from www.python.com, Cygwin (a data storage) c:\program and Python 2.5 were simultaneously downloaded from www.cygwin.com, Molecular graphics laboratory (MGL) tools and AutoDock 4.2 was downloaded from www.scripps.edu, Discovery studio visualizer 2.5.5 was downloaded from www.accelerys.com, Molecular orbital package (MOPAC), ChemSketch was downloaded from www.acdlabs.com. Online smiles translator was carried out using cactus.nci.nih.gov/translate/.

2.2 Chemicals required: Allopurinol, xanthine, xanthine oxidase from bovine milk source and terpenoids like bisabolol, β-caryophyllene, limonene, α- terpinene were purchased from Sigma Aldrich, USA. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

2.3 In silico docking studies: The optimized ligand molecules were docked into refined xanthine oxidase model using "LigandFit" in the Autodock 4.2[14]. We employed the Lamarckian genetic algorithm (LGA) for ligand conformational searching, which is a hybrid of a genetic algorithm and a local search algorithm. This algorithm first builds a population of individuals (genes), each being a different random conformation of the docked molecule. Each individual is then mutated to acquire a slightly different translation and rotation and the local search algorithm then performs energy minimizations on a user-specified proportion of the population of individuals. The individuals with the low resulting energy are transferred to the next generation and the process is then repeated. The

algorithm is called Lamarckian because every new generation of individuals is allowed to inherit the local search adaptations of their parents[15].
An extended PDB format, termed as PDBQT file was used for coordinate files which includes atomic partial charges. AutoDock Tools was used for creating PDBQT files from traditional PDB files[16]. Crystal structure of xanthine oxidase enzyme was downloaded from the Brookhaeven protein data bank (Fig. 1).

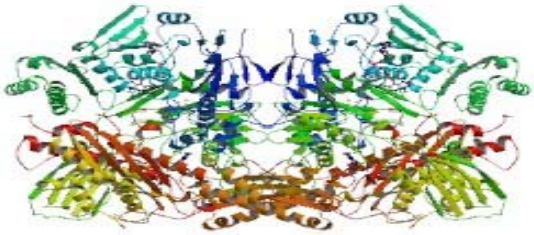


Fig. 1 Xanthine oxidase enzyme from Brookhaeven protein data bank (1FIQ)

The flavonoid ligands like bisabolol, β-caryophyllene, limonene, α- terpinene and allopurinol were built using ChemSketch and optimized using "Prepare Ligands" in the AutoDock 4.2 for docking studies.

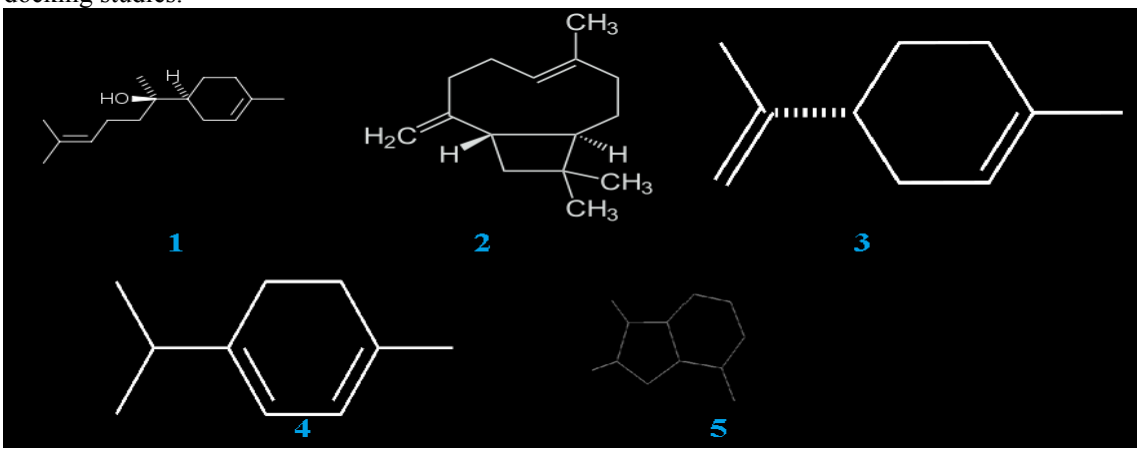


Fig. 2 The optimized ligand molecules (**1** bisabolol, **2** β-caryophyllene, **3** limonene, **4** α- terpinene, and **5** allopurinol)

The preparation of the target protein 1FIQ with the AutoDock Tools software involved adding all hydrogen atoms to the macromolecule, which is a step necessary for correct calculation of partial atomic charges. Gasteiger charges are calculated for each atom of the macromolecule in AutoDock 4.2 instead of Kollman charges which were used in the previous versions of this program. Three-dimensional affinity grids of size 277 × 277 × 277 Å with 0.6 Å spacing were centered on the geometric center of the target protein and were calculated for each of the following atom types: HD, C, A, N, OA, and SA, representing all possible atom types in a protein. Additionally, an electrostatic map and a desolvation map were also calculated[15].
Rapid energy evaluation was achieved by precalculating atomic affinity potentials for each atom in the ligand molecule. In the AutoGrid procedure, the target enzyme was embedded on a three dimensional grid point[8]. The energy of interaction of each atom in the ligand was encountered.
We have selected important docking parameters for the LGA as follows: population size of 150 individuals, 2.5 million energy evaluations, maximum of 27000 generations, number of top individuals to automatically survive to next generation of 1, mutation rate of 0.02, crossover rate of

0.8, 10 docking runs, and random initial positions and conformations. The probability of performing local search on an individual in the population was set to 0.06.

AutoDock was run several times to get various docked conformations, and used to analyze the predicted docking energy. The binding sites for these molecules were selected based on the ligand-binding pocket of the templates[17]. AutoDock Tools provide various methods to analyze the results of docking simulations such as, conformational similarity, visualizing the binding site and its energy and other parameters like intermolecular energy and inhibition constant. For each ligand, ten best poses were generated and scored using AutoDock 4.2 scoring functions[16].

2.4 In vitro xanthine oxidase inhibitory activity: The assay mixture consisted of 1ml of the test compound (5 – 100 µg/ml), 2.9 ml of phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.1 units/ml in phosphate buffer, pH 7.5), which was prepared immediately before use. After preincubation at 25ºC for 15 min, the reaction was initiated by the addition of different concentration (5 – 100 µg/ml) of the substrate solution. The assay mixture was incubated at 25ºC for 30 min. The reaction was stopped by adding 1 ml of 1 N HCl and the absorbance was measured at 290 nm using an UV spectrophotometer[18, 19]. Allopurinol (5 – 100 µg/ml) was used as the standard. The percentage inhibition was calculated by,

Percentage inhibition = { (A-B) – (C-D)}/(A-B) X 100

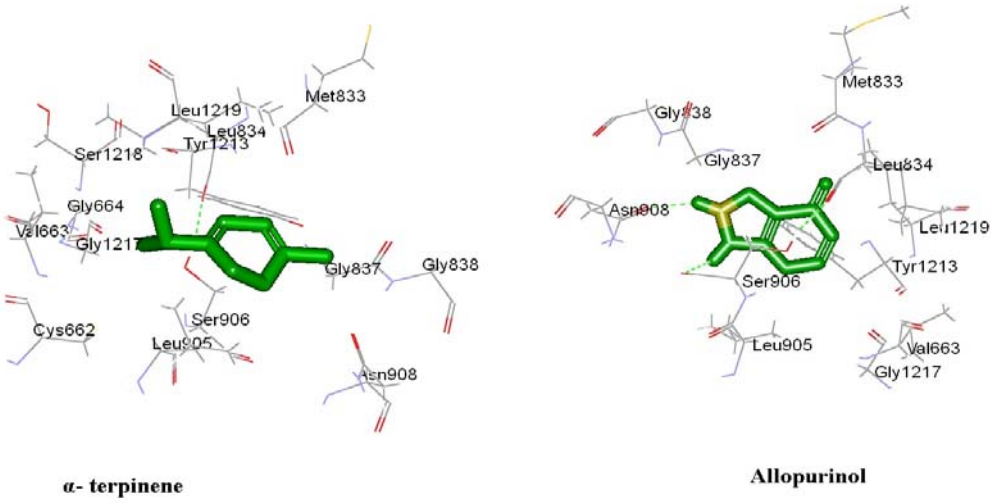
where A is the activity of the enzyme without the compound, B is the control of A without the compound and enzyme, C and D are the activities of the compound with or without XO respectively. The assay was done in triplicate and IC_{50} values were calculated from the percentage inhibition[20].

2.5 Enzyme kinetics studies: Lineweaver – Burk plot analysis was performed to determine the mode of inhibition of flavonoids and compared with allopurinol. The assay was carried out in the presence or absence of flavonoids with varying concentrations of xanthine as the substrate, employing the xanthine oxidase assay as mentioned earlier. Lineweaver – Burk transformed values were plotted to determine the mode of enzyme inhibition[21, 22].

3. Results and Discussion

***3.1 In silico* docking studies:** The virtual screening analysis was performed by the use of AutoDock 4.2. The docking poses are ranked according to their docking scores and both the ranked list of docked ligands and their corresponding binding poses[15]. In Fig. 3, docked pose of xanthine oxidase enzyme with ligand clearly demonstrates the binding positions of the α- terpinene and the standard with the enzyme.

Amino acid residues closer to the α- terpinene ligand binding site are Tyr1213, Leu915, Leu1219, Ser1218, Gly1217, Cys662, Val663, Gly664, Leu834, Met833, Ser906, Leu905, Gly837, Gly838 and Asn908. Amino acid residues closer to the ligand binding site are Met833, Leu834, Leu1219, Tyr1213, Val663, Gly1217, Leu905, Ser906, Gly837, Gly838 and Asn908.



In Fig. 3, docked pose of xanthine oxidase enzyme with α- terpinene and the standard

As shown in table 1, flavonoids showed binding energy ranging from -7.60 kcal/mol to -5.90 kcal/mol. All the selected compounds had lesser binding energy when compared to the standard allopurinol (-4.47 kcal/mol). This proves that terpenoids consist of potential xanthine oxidase inhibitory binding sites when compared to the standard.

In addition, two other parameters like inhibition constant (K_i) and intermolecular energy were also determined. As shown in table 2, flavonoids showed inhibition constant ranging from 2.66 µM to 47.68 µM. All the selected compounds had lesser inhibition constant when compared to the standard (529.73 µM). Inhibition constant is directly proportional to binding energy. We found a decrease in inhibition constant of all the selected terpenoids with a simultaneous decrease in the binding energy. When the binding energy of the compound decreases, there is an increase in activity. Thus, the xanthine oxidase inhibitory activity of the terpenoids were found to be higher compared to allopurinol.

As shown in table 3, flavonoids showed intermolecular energy ranging between -8.80 kcal/mol to -6.19 kcal/mol which was lesser when compared to the standard (-4.47 kcal/mol). Intermolecular energy is also directly proportional to binding energy. We found a decrease in intermolecular energy of all the selected compounds with a simultaneous decrease in the binding energy. This result further proved the xanthine oxidase inhibitory activity of all the selected terpenoids.

Based on the docking studies, the activity of the selected compounds was in order of α- terpinene > bisabolol > β-caryophyllene > limonene > allopurinol. On the basis of the above study, α- terpinene, bisabolol, β-caryophyllene and limonene possess the highest xanthine oxidase inhibitory sites when compared to that of the standard. This may due to the structural properties of the compounds.

3.2 In vitro xanthine oxidase inhibitory activity: An increase in the level of xanthine oxidase causes renal stone formation, ischemic myocardium and free radical induced diseases[23, 24]. Recent findings suggest that the occurrence of gout is increasing worldwide, possibly due to the changes in dietary habits like intake of foods rich in nucleic acid. Hypouricemic agents are commonly employed for the treatment of chronic gouty arthritis which includes xanthine oxidase inhibitors and uricosuric agents[25,26].

All the selected terpenoids exhibited a dose dependent inhibition of XO enzyme activity. It is proved that inhibition of XO resulted in a decreased production of uric acid, which was measured by UV spectroscopy. All the compounds demonstrated XOI activity at a concentration of 100 µg/ml, showing an inhibition greater than 50%. Their IC_{50} values were found to be ranging between 34.70±1.06 µg/ml to 68.45±1.07 µg/ml. Bisabolol was found to have highest activity as the IC_{50} value was found to be 34.70±1.06 µg/ml. All the remaining compounds showed IC_{50} values below 100 µg/ml. The XOI activity of the all the selected compounds was in order of allopurinol > bisabolol > limonene > β-caryophyllene > α- terpinene. These results were compared with that of the standard allopurinol, which showed the IC_{50} value of 8.48± 0.17 µg/ml. The *in vitro* results coincide with the virtual screening analysis which further proves the inhibitory potential of the terpenoids against the enzyme.

3.3 Enzyme kinetic studies: The enzyme kinetic studies were performed using Lineweaver Burk plot analysis. The terpenoids like Limonene, α- terpinene and allopurinol were found to be competitive inhibitors of xanthine oxidase due to their increase in substrate concentration and larger Km value in the Lineweaver Burk plot analysis (Fig.4). The remaining selected terpenoids like bisabolol and β caryophyllene were found to be non competitive inhibitors of xanthine oxidase.

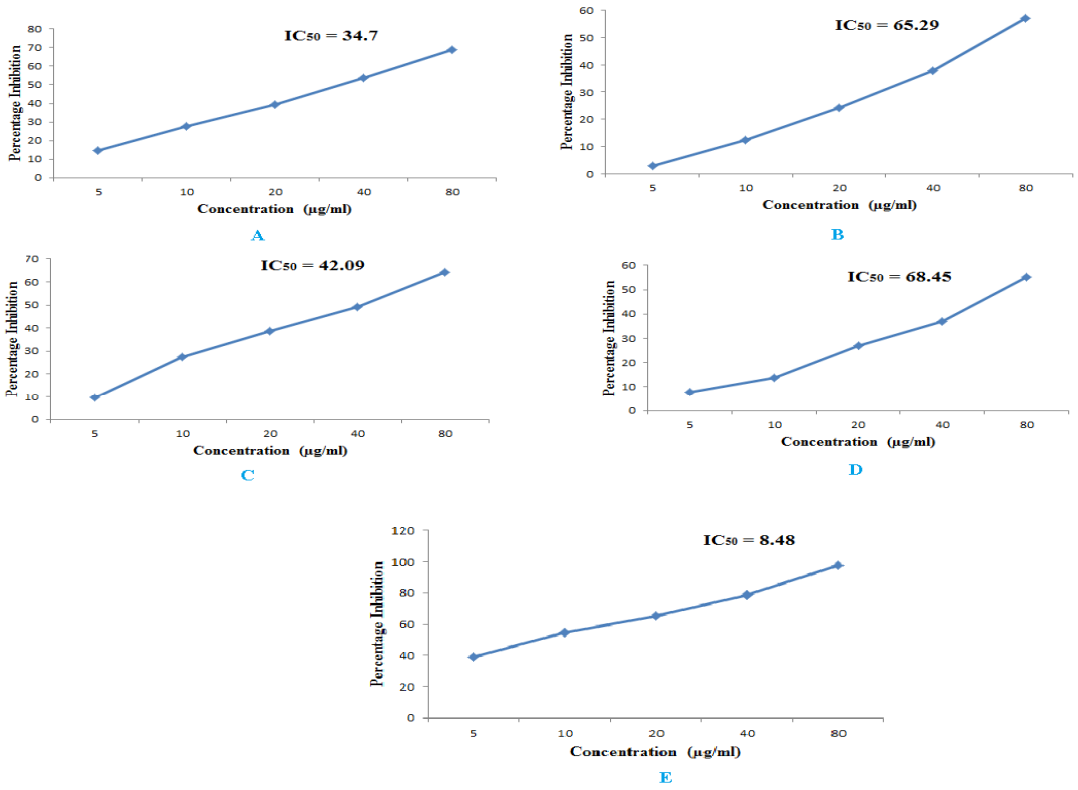


Fig. 4 Lineweaver-Burk plots of terpenoids (A bisabolol, B β-caryophyllene, C limonene, D α-terpinene, and E allopurinol)

Conclusion

The results of the present study clearly demonstrated the xanthine oxidase inhibitory activity of the selected terpenoids by *in silico* docking analysis and *in vitro* assay. *In silico* docking analysis is actually an added advantage to screen the xanthine oxidase inhibition. These results clearly indicate that terpenoids especially, bisabolol, β-caryophyllene, Limonene and α- terpinene have excellent binding interactions with xanthine oxidase. In the enzyme kinetic studies, bisabolol, β-caryophyllene showed non competitive and Limonene, α- terpinene and allopurinol showed competitive type of enzyme inhibition. It can be concluded that terpenoids could be a promising remedy for the treatment of gout and related inflammatory disorders. Further investigations on the above compounds and *in vivo* studies are necessary to develop potential chemical entities for the prevention and treatment of gout and related inflammatory disorders.

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Table 1. Binding energies of the compounds based on their rank

COMPOUNDS	Binding energies of the compounds based on their rank (kcal/mol)									
	1	2	3	4	5	6	7	8	9	10
Bisabolol	-7.33	-6.53	-6.43	-6.22	-6.12	-6.12	-6.11	-6.06	-5.95	-6.06
β-caryophyllene	-5.91	-5.90	-5.90	-5.89	-5.78	-5.78	-5.76	-5.79	-5.25	-5.25
Limonene	-5.90	-5.65	-5.56	-5.52	-5.52	-5.42	-5.34	-5.32	-5.17	-5.00
α- terpinene	-7.60	-7.40	-7.25	-6.70	-7.18	-7.13	-7.16	-5.79	-5.58	-5.36
Allopurinol	-4.47	-4.47	-4.46	-4.46	-4.45	-4.20	-4.09	-4.09	-3.99	-3.87

Table 2. Inhibition Constant of the compounds based on their rank

COMPOUNDS	Inhibition Constant of the compounds based on their rank (μM, mM*)									
	1	2	3	4	5	6	7	8	9	10
Bisabolol	4.25	16.43	19.50	27.49	32.84	32.86	33.17	36.01	43.33	36.25
β-caryophyllene	46.50	47.05	47.51	47.76	57.79	57.87	59.60	56.64	140.79	141.30
Limonene	47.68	71.76	84.03	90.31	90.60	107.25	121.84	125.59	162.42	215.41
α- terpinene	2.66	3.77	4.81	12.19	5.46	5.94	5.66	57.27	81.19	117.43
Allopurinol	529.73	534.14	541.00	541.30	545.56	830.85	1.01*	1.01*	1.18*	1.45*

Table 3. Intermolecular energies of the compounds based on their rank

COMPOUNDS	Inter molecular energies of the compounds based on their rank									
	1	2	3	4	5	6	7	8	9	10
Bisabolol	-8.82	-8.02	-7.92	-7.71	-7.61	-7.61	-7.60	-7.55	-7.44	-7.55
β-caryophyllene	-5.91	-5.90	-5.90	-5.89	-5.78	-5.78	-5.76	-5.79	-5.25	-5.25
Limonene	-6.19	-5.95	-5.86	-5.82	-5.81	-5.71	-5.64	-5.62	-5.47	-5.30
α- terpinene	-8.80	-8.59	-8.45	-7.90	-8.37	-8.32	-8.35	-6.98	-6.77	-6.55
Allopurinol	-4.47	-4.47	-4.46	-4.46	-4.45	-4.20	-4.09	-4.09	-3.99	-3.87

Table 4. *In vitro* xanthine oxidase inhibitory activity of the selected compounds

COMPOUNDS	Percentage Inhibition					IC_{50} μg/ml
	Concentration (μg/ml)					
	5	20	40	60	80	
Bisabolol	14.80±1.35	27.76±1.53	39.51±0.51	53.73±0.91	68.98±0.15	34.70±1.06*
β-caryophyllene	2.81±0.94	12.17±0.95	24.20±0.81	37.87±1.35	57.05±0.29	65.29±1.20*
Limonene	9.53±1.49	27.45±0.54	38.50±0.22	49.21±0.43	64.32±0.32	42.09±1.06*
α- terpinene	7.73±0.93	13.64±1.92	26.88±1.32	37.01±1.19	48.52±0.27	68.45±1.07*
Allopurinol	39±0.16	54.76±0.76	65.42±0.33	78.56±0.13	97.76±0.03	8.48± 0.17

Values are mean±S.E.M. of three parallel measurements
*P < 0.01 considered significant when compared to allopurinol (Oneway ANOVA followed by Dunnett's test)