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RESEARCH ARTICLE

Quercetin Based Standardization of Polyherbal Anti-Gout Remedy and Its Molecular Docking Study Against Anti-Gout and Anti-Inflammatory Protein Targets

Ayisha SHAUKAT[*°], Khalid HUSSAIN[**]

Quercetin Based Standardization of Polyherbal Anti-Gout Remedy and Its Molecular Docking Study Against Anti-Gout and Anti-Inflammatory Protein Targets

SUMMARY

A five-herb-containing traditional homemade medicine is extensively used to treat gout but has not been standardized for quercetin content. Therefore; the current study describes a reversed-phase liquid chromatographic method for quercetin determination in traditional herbal remedy. The elution was carried out using aqueous 2.0% acetic acid, acetonitrile, and tetrahydrofuran (55:40:5, V/V/V) as mobile phase at a flow rate of 0.8 mL/min, and detection was performed using diode array detector operated at 370 nm. The detector's response was linear in the range investigated (2.5-160.0 µg/mL) with R2 = 0.996. The results of recovery (98.26-103.22%, SD<5%), intraday accuracy and precision (94.68-104.08%, RSD<5%), and interday accuracy and precision (92.31-104.92%, RSD<5%) showed that the method was reliable, repeatable and reproducible, hence may be used for determination of quercetin in herbal remedy. The medicine contained 0.2425 mg/g quercetin. The molecular interactions of this marker compound were also studied against anti-gout and anti-inflammatory protein targets. Hence, the developed RP-HPLC method may be applied to standardize anti-gout medicine for quercetin content. Moreover, the molecular interactions help in the understanding of the underlying mechanism of action of this marker compound against gout.

Keywords: *Polyherbal, quercetin, RP-HPLC, standardization*

Anti-gut Poliherbal İlacın Kersetin Tabanlı Standardizasyonu ve Anti-gut ve Anti-inflamatuvar Protein Hedeflerine Karşı Moleküler Yerleştirme Çalışması

ÖZ

Beş bitki içeren geleneksel ilaç, gut tedavisinde yaygın olarak kullanılmaktadır, ancak kersetin içeriği bakımından standardize edilmemiştir. Bu nedenle, mevcut çalışma, geleneksel bitkisel ilaçta kersetin tayini için ters fazlı sıvı kromatografik bir yöntemi açıklamaktadır. Elüsyon, mobil faz olarak sulu %2.0 asetik asit, asetonitril ve tetrahidrofuran (55:40:5, V/V/V) kullanılarak 0.8 mL/dk akış hızında gerçekleştirilmiş ve 370 nm'de çalıştırılan diyot dizi dedektörü kullanılarak tanımlama yapılmıştır. Dedektörün yanıtı, R2 = 0.996 ile araştırılan aralıkta (2.5-160.0 µg/mL) doğrusaldır. Geri kazanım (%98,26-103,22, SD<5), gün içi doğruluk ve kesinlik (%94,68-104,08, RSD<%5) ve günler arası doğruluk ve kesinlik (%92,31-104,92, RSD<%5) sonuçları, yöntemin güvenilir, çoğaltılabilir ve tekrarlanabilir olduğunu, dolayısıyla bitkisel ilaçlarda kersetin tayini için kullanılabileceğini göstermiştir. İlaç 0.2425 mg/g kersetin içermektedir.. Bu işaretleyici bileşiğin moleküler etkileşimleri gut önleyici ve iltihap önleyici protein hedeflerine karşı da incelenmiştir. Bu nedenle, geliştirilen RP-HPLC yöntemi, kersetin içeriği bakımından standardize edilmiş gut önleyici ilaç üretmek için kullanılabilir. Ayrıca, moleküler etkileşimler, bu işaretleyici bileşiğin gut hastalığına karşı altta yatan etki mekanizmasının anlaşılmasına yardımcı olacaktır.

Anahtar Kelimeler: *Poliherbal, kersetin, RP-HPLC, standardizasyon*

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INTRODUCTION

A folklore polyherbal medicine reported in the literature is being extensively used for curing gout (Shaukat et al., 2020). Herbal formulations are inconsistent in terms of chemical constituents due to various genetic, growing, harvesting, and environmental factors. Hence, for a reproducible claim, herbal product needs to be standardized prior to pharmacological evaluation. The markers and characteristics compounds found in the ingredients of the traditional remedy can be used as analytical markers to develop various analytical methods for herbal product standardization (Li et al., 2008).

Quercetin, a flavonoid, found in the ingredients of the remedy, is also reported to have xanthine oxidase inhibitory potential (Zhu et al., 2004; Lin et al., 2015; Nile et al., 2017). Hence, it was selected as an analytical standard to develop HPLC method for standardization of the remedy. The literature review indicated various reversed-phase HPLC methods for the determination of quercetin in extracts, serum, urine, tea, and different matrices using diode array detector, solid-phase extraction and UV detection, electrochemical detection, and coulometric electrode array detection (Careri et al., 2003; Ishii et al., 2003; Fasolo et al., 2007; Goo et al., 2009; Phani et al., 2010; Liu et al., 2011). Most of the methods were expensive, time-consuming and laborious. Hence, there is a need to develop a simple and specific RP-HPLC method for quantification of quercetin which can easily be performed in less equipped laboratories for standardization and routine quality control of the remedy.

Molecular docking is a commonly used structure-based drug design strategy due to its extensive applications in finding molecular interactions and binding energy. Docking uses a scoring function that ranks candidate dockings by searching high-dimensional spaces effectively (Meng et al., 2011; Kalyaanamoorthy and Chen, 2011). Xanthine oxidase (XO) is an essential enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid, which is excreted by kidneys. Excessive production and, or inadequate excretion of uric acid results in hyperuricemia and gout (Borges et al., 2002). Quercetin inhibits xanthine oxidase due to its hydrophobic interaction with the enzyme and binding of the 3-hydroxyl group on benzopyrene ring of quercetin with isoalloxazine ring of FAD domain of the enzyme (Lin et al., 2002; Rasoulzadeh et al., 2009; Nessa et al., 2010). Prostaglandins are generated from arachidonate by the action of cyclo-oxygenase (COX) isoenzymes, and their biosynthesis is blocked by nonsteroidal anti-inflammatory drugs (NSAIDs). Prostaglandins play a vital role in the generation of the inflammatory response, their biosynthesis is significantly increased in inflamed tissue, and they contribute to the development of the cardinal signs of acute inflammation (Ricciotti and Fitzgerald, 2011). Quercetin inhibits COX-2, thus inhibiting the release of inflammatory mediators involved in inflammation, thus, inhibits carrageenan-induced inflammation in experimental rats (Morikawa et al., 2003). The inhibition of carrageenan-induced hind paw edema of rats is well correlated with inhibition of such inflammatory mediators as reported in literature (Shaukat et al., 2021). Phospholipase-A_2 hydrolyze cell membrane phospholipids (ester bonds) to produce arachidonic acid and fatty acid (lysophosphatidylcholine and lysophospholipids), playing an essential role in the production of inflammatory lipid mediators, mainly eicosanoids. Hence, they are considered pro-inflammatory enzymes, and their inhibition is regarded as a desirable therapeutic target (Yedger et al., 2006). Quercetin selectively inhibits phospholipase A_2, thus inhibiting mediators of inflammation (Lindal and Tagesson, 1997).

The present studies are performed to simulate the wet lab results with dry lab studies, hence, a freely available software was used for the blind type of docking to evaluate all possible active sites as well as allosteric sites. Therefore, the present study aimed to quantify quercetin in herbal remedy and to under-

stand its molecular interaction with anti-gout and anti-inflammatory protein targets.

MATERIAL AND METHODS

Chemicals and solvents

The chemicals and solvents used in the current study are acetonitrile, methanol, tetrahydrofuran, quercetin, acetic acid and sodium acetate (Merck, Germany). The double distilled water prepared in-house was used, where required.

Instruments

Double beam UV/Visible spectrophotometer (Model-2550, Shimadzu Scientific Instruments, USA, equipped with Operating system UV Probe 2.21), Fourier Transform Infrared Spectrophotometer (IR Tracer-100, Shimadzu Japan). A liquid chromatography system (Agilent Technologies, 1200 series, Germany) equipped with an isocratic pump (G1310A), auto-sampler (G1329A), thermostatically controlled column oven (G1316A), and diode array detector (G1315B) were used in the current study. Other equipment used included a pH meter (WTW series, Ino lab) and ultrasonicator (Memmert, Germany).

Preparation of anti-gout remedy

The anti-gout remedy was prepared by using method reported in the literature (Shaukat et al., 2020).

Preparation of standard solutions

A stock solution of quercetin (1.0 mg/mL) was prepared in HPLC-grade methanol. Working standard solution having concentration range of 2.5-160 µg/mL was prepared by diluting the standard stock solution with the mobile phase.

Method development

A volume (20 µl) was eluted by the isocratic mobile phase comprising 2.0% acetic acid, acetonitrile, and tetrahydrofuran (55:40:5, *V/V/V*) at 0.8 mL/min flow rate through the C_{18} column (Agilent 5 TC-C_{18} (2) 250×4.6 mm) that was maintained at 35°C. The detection was carried out using DAD, 370 nm detection wavelength, and 360 nm reference beam. The chromatogram obtained was used to determine system suitability.

System suitability

The system suitability was ensured by determining the number of theoretical plates (N), height equivalent to theoretical plate (HETP), capacity factor (k´), tailing factor, and peak asymmetry.

Method validation

Linearity, Beer's range, limit of detection and quantification

The working standard solution having a concentration range of 2.5-160 µg/mL was analyzed in triplicate. The plot between concentration and peak area was constructed, and linearity was evaluated visually by applying the linear regression equation. The correlation of data points was assessed by the correlation coefficient.

The sensitivity, Limit of detection (LOD) and Limit of quantification (LOQ), were determined using the statistical method. Briefly, five standard solutions of quercetin (5.0-80.0 µg/mL) were analyzed in quintuplicate. The standard calibration curve was constructed to determine the mean slope (S) and standard deviation of intercepts (σ) to determine LOD and LOQ using Equations 1 and 2, respectively.

Recovery

For recovery, the dried herbal extract (20 mg) was spiked with 1 mL of working standard solutions containing quercetin (5.0, 10.0 and 20.0 µg/mL). Un-spiked samples were treated in a similar procedure to prepare respective blanks. The spiked and un-spiked samples were analyzed in triplicate, the peak corresponding to standard was identified, and the peak area was used to determine the amount of quercetin using the calibration curve. The calculated amount was then compared with the spiked amount to assess recovery.

Intra-day and inter-day accuracy and precision

Intra-day and inter-day accuracy and precision were determined using each of the three mixed standard solutions used for recovery studies which were analyzed six times in a single day and once daily for six consecutive days, respectively. Accuracy was determined by quantification of each standard from the respective standard curve, constructed on each day, whereas the RSD of the six readings was taken as precision.

Robustness

The working standard solution of quercetin was analyzed by changing mobile phase pH (± 0.1), detection wavelength (± 2 nm) and column temperature (± 2°C).

Quantification of quercetin in polyherbal anti-gout remedy

The sample for HPLC analysis was prepared by acid hydrolysis of the herbal remedy using the method reported in the literature (Ewais et al., 2016). The sample solution having a concentration of 20 mg/mL was prepared for quantification of quercetin in traditional herbal remedy. The amount of quercetin was calculated using linear regression equation obtained from the standard calibration curve of quercetin.

Molecular docking studies

The 1-Click Docking Mcule and UCSF Chimera 1.12 Software were used for docking and determining the hydrogen-bonding affinity of the ligands against xanthine oxidase, prostaglandin G_1/H_1 synthase, prostaglandin G_2/H_2 synthase and phospholipase A_2.

Quercetin was used as ligand. The data input was in the form of SMILES of the ligands, taken from the PUB-Chem database. The SMILES code of quercetin is given as (C1=CC(=C(C=C1C2=C(C (=O) C3=C(C=C(C=C3O2)O)O)O)O)O), allopurinol is C1=NNC2=C1C(=O)NC=N2 and diclofenac is 1=CC=C(C(=C1)CC(=O)O)NC2=C(C=CC=C2Cl) Cl. The PDB file of each ligand was evaluated using UCSF Chimera 1.12 to find hydrogen bonding and bond lengths.

Interpretation of molecular docking

The least binding energies (kcal/mol) were noted for the ligand-protein interaction. The amino acids involved in interaction and bond angles were noted. The best possible binding pose showing hydrogen bonding of ligand was explored.

RESULTS AND DISCUSSION

In plants, phenolic compounds often occur as glycosides and sugar moieties affect their elution pattern. Moreover, the presence of the type of sugar makes the selection of standard difficult. Therefore, in the present study, the sample of polyherbal anti-gout remedy was subjected to acid hydrolysis to break the glycosidic bond. The aglycone-containing fraction was then extracted by partitioning with ethyl acetate. Ethyl acetate was not miscible with water, so it was removed and the samples were made with the mobile phase. This extraction produced aglycone-rich samples, free from interfering substances. Moreover, it helped to make the method more specific and sensitive.

Quercetin exhibits maximum absorbance at two wavelengths (280 nm due to benzoyl ring and 370 nm due to cinnamyl ring) which can be used to develop UV absorbance-based methods (Duan, 2014). However, it exhibits intrinsic absorption at 370 nm due to functional groups in the flavonoid ring giving a broad absorption peak at this wavelength (Yao et al., 2004; Dmitrienko et al., 2012). Therefore, in the present study, 370 nm wavelength was selected to detect quercetin using diode array detection. In plants, quercetin is also found in the form of glycosides (rutin); hence, the samples of polyherbal anti-gout remedy were prepared like the determination of phenolic and polyphenolic compounds.

The chromatogram of the standard quercetin solution obtained using the optimized chromatographic condition is given in Figure 1. These results showed that the peak of the standard appeared at 5.480 ± 0.1 min. Moreover, the peak was symmetrical/Gaussian which could be used to determine system suitability.

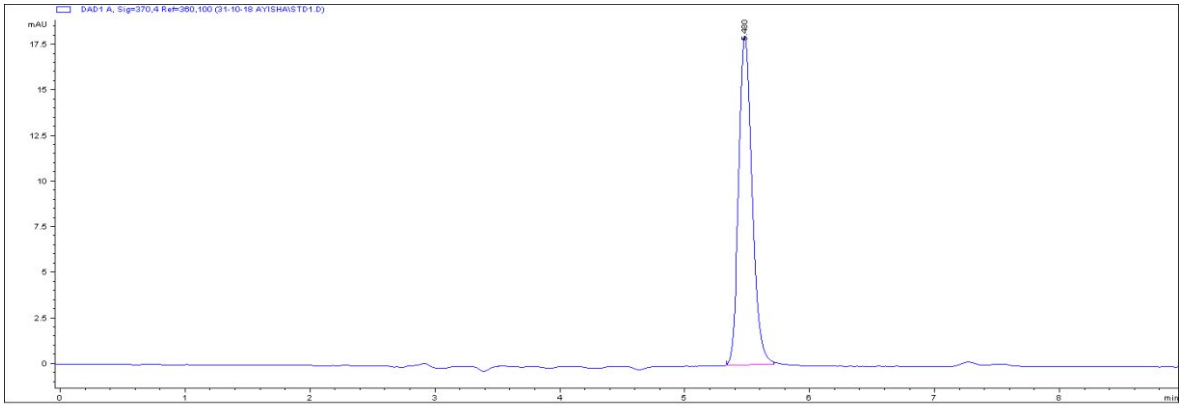

Figure 1. HPLC chromatogram of standard marker (quercetin)

The system suitability parameters are given in Table 1. The results were within the specified limits, which indicated that the chromatographic system and conditions were suitable to be used for quantitative purposes. Hence, the method can be validated for the standardization of anti-gout remedy.

Table 1. System suitability parameters calculated from chromatogram of quercetin.

Parameters	Values	Reference values
Capacity(retention) factor k´	4.48	K ≥ 2
Peak asymmetry/Tailing factor As	1.0	T ≤ 2
Number of theoretical plates N	12012.16	N > 2000
Height equivalent to theoretical plate	20.81	The smaller the value, the higher the efficiency of the column

The plot of concentration versus peak area of quercetin is shown in Figure 2. These results indicated that the method was linear in the concentration range investigated (2.5- 160 µg/mL).

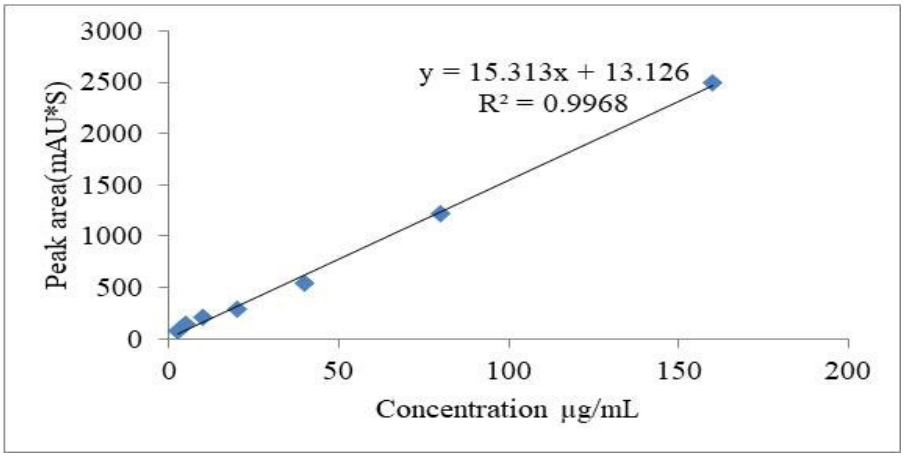

Figure 2. A plot of concentration versus peak area of quercetin.

The sensitivity of the method – LOD and LOQ – was determined statistically using the standard deviation of intercepts and the mean slope (S) of standard calibration curves (n=5), which was found to be 0.36 and 1.10 µg/mL, respectively (Table 2). These results indicate that the method can be used to quantify this marker even at the sub-microgram level.

Table 2. Limit of detection (LOD) and limit of quantification (LOQ) of quercetin by RP-HPLC

Standard curve	Concentration(µg/mL)	Linear regression equation	Slope	Intercept
1	5.0-80.0	Y=14.299x+19.925	14.299	19.925
2	5.0-80.0	Y=13.126x+22.383	13.126	22.383
3	5.0-80.0	Y=13.067x+21.023	13.067	21.023
4	5.0-80.0	Y=12.996x+19.819	12.996	19.819
5	5.0-80.0	Y=12.928x+18.454	12.928	18.454
Mean slope (S) = 13.283				
Standard deviation (SD) = 1.46				
LOD=3.3SD/S = 0.36 µg/mL				
LOQ=10SD/S = 1.10 µg/mL				

The mean recovery of spiked samples with three different concentrations (5.0, 10.0 and 20.0 µg/mL) are given in Table 3. The recovery was found to be ranging from 98.26 to 103.22%, with (SD) less than 5%. These results were within acceptable limits, which showed that the developed method was reliable. The results of intra- and inter-day accuracy and precision are shown in Table 3. The Intra- and Intra-day accuracy for quercetin was found to be (94.68-104.08%; 92.31-104.92%) with the relative standard deviation of less than 5%, which indicated that the developed method was repeatable and reproducible. The chromatograms of the remedy showed that the peak of quercetin was well resolved without the interference from any other component. Hence, the developed method is specific for the determination of quercetin.

Table 3. Recovery, Intraday, inter-day accuracy and precision of the HPLC method for the determination of quercetin (n = 6)

Concentration (µg/mL)	Mean recovery (%)± SD	Intra-day analysis		Inter-day analysis	
		Accuracy %	Precision RSD	Accuracy %	Precision RSD
5.0	102.27±0.057	104.08	1.97	104.92	2.12
10.0	103.22±0.173	97.05	0.52	98.03	1.90
20.0	98.26±0.152	94.68	0.22	92.31	0.46

The developed method was robust since slight variation in mobile phase pH (± 0.1), column temperature (± 2°C) and detection wavelength (± 2 nm) did not affect the chromatographic resolution.

The chromatogram of the polyherbal anti-gout remedy is given in Figure 3. The peak of the standard was identified by comparing retention time, and the peak area was used to quantify the marker in the polyherbal anti-gout remedy. The amount of quercetin in the anti-gout remedy was found to be 0.2425 mg/g.

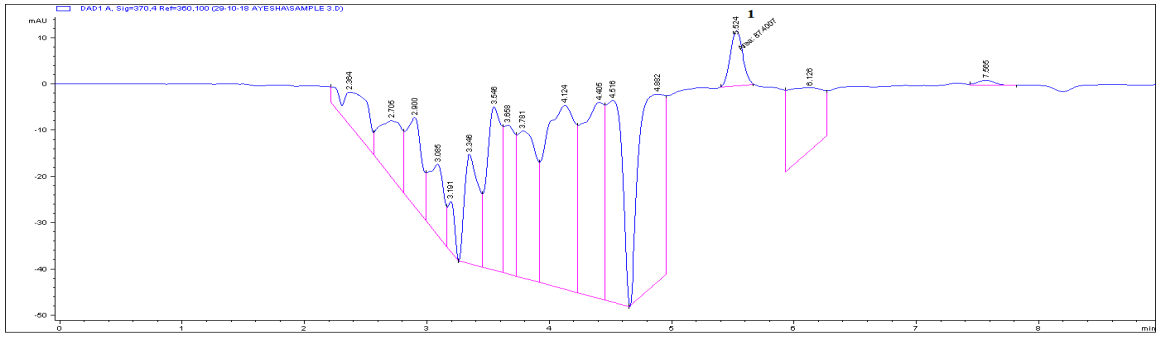

Figure 3. Chromatogram of polyherbal anti-gout remedy; Quercetin (1)

In the present study, we have used quercetin as an analytical marker to standardize a polyherbal anti-gout remedy. The sensitivity of the method was found to be more than the method reported in the literature Sladkovsky et al. (2001), and lesser than some of the studies (Chen and Xiao, 2010; Savic et al., 2013).

Amino acid residues of xanthine oxidase ALA 1054.D, SER 1057.D formed two hydrogen bonds with quercetin and four hydrogen bonds with allopurinol(GLY 772.D, PHE 773.D, GLN 1169.D). The least binding energies of -9.2 and -6.4 were noted for quercetin and allopurinol, respectively (Figure 4 and Figure 5).

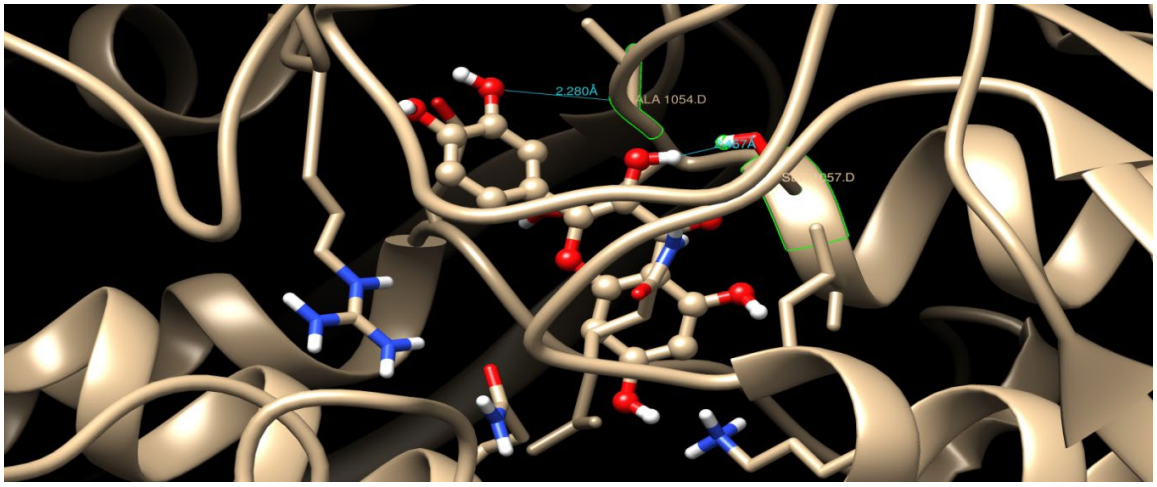

Figure 4. Interaction of quercetin with xanthine oxidase

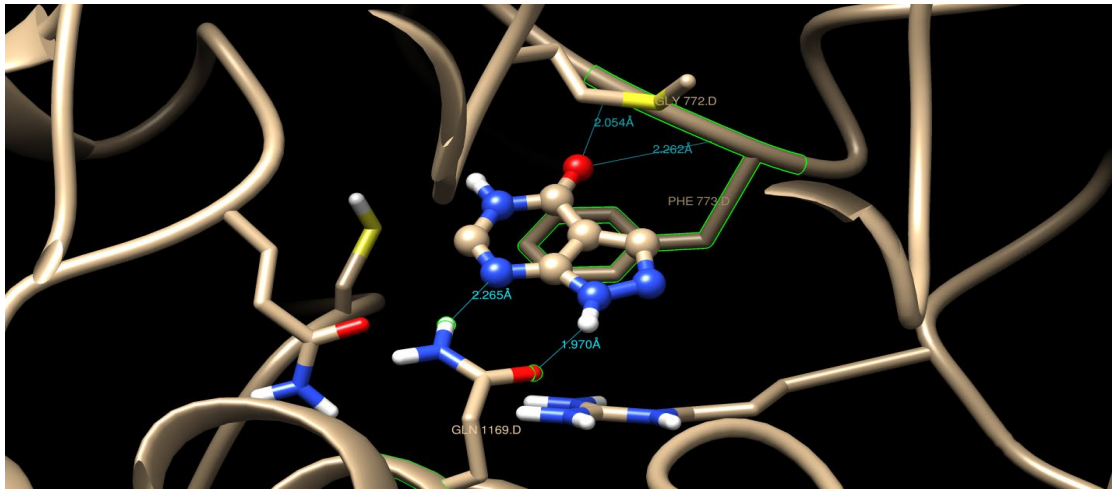

Figure 5. Interaction of Allopurinol with xanthine oxidase

The lower values for the binding energy showed the good binding affinities of ligands with the enzymes. The interaction occurs through the formation of hydrogen bonding between hydroxyl groups of quercetin and allopurinol and catalytic residues of the binding sites, which results in the formation of a conjugated pi-system responsible for stabilizing interaction with the active site.

The docking of prostaglandin G_2/H_2 with quercetin showed that two hydrogen bonds were involved in the binding interaction of SER 499.C and THR 175.C with quercetin (Figure 6). Two hydrogen bonds were involved in the binding interaction of TYR 354.C and SER 499.C with diclofenac (Figure 7). The docking studies of enzyme prostaglandin G_1/H_1 have shown a strong binding interaction via formation of two hydrogen bonds with quercetin and one hydrogen bond with diclofenac (Figure 8 and Figure 9). The docking studies of enzyme phospholipase A_2 have shown a strong binding interaction via formation of two hydrogen bonds, each with quercetin and diclofenac (Figure 10 and Figure 11).

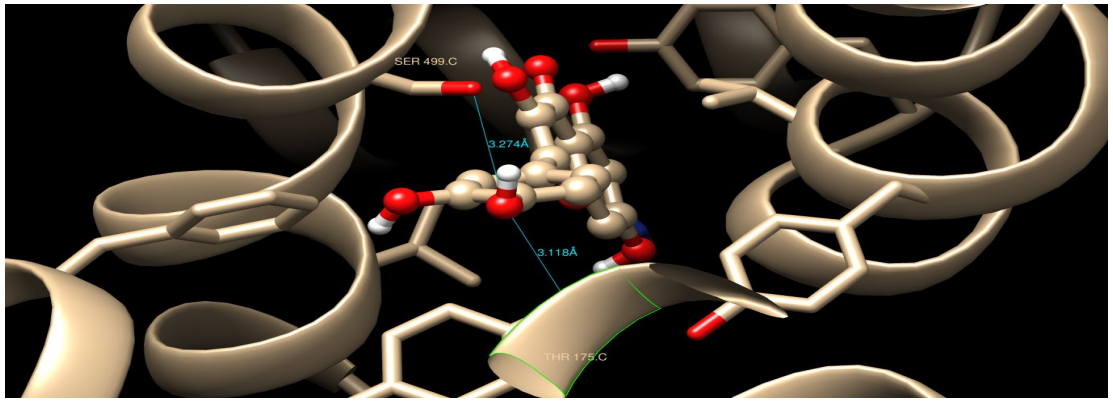

Figure 6. Interaction of quercetin with prostaglandin synthase G_2/H_2

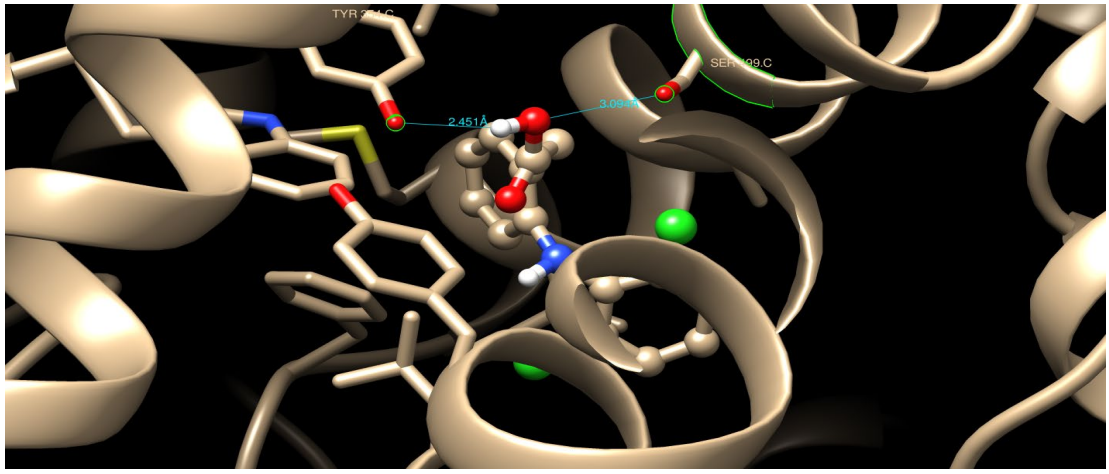

Figure 7. Interaction of diclofenac with prostaglandin G_2/H_2

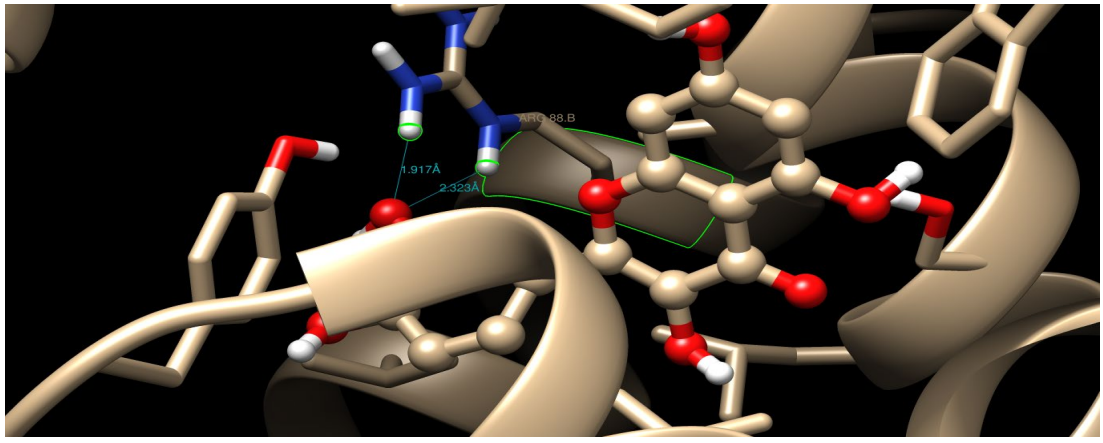

Figure 8. Interaction of quercetin with prostaglandin G_1/H_1 synthase

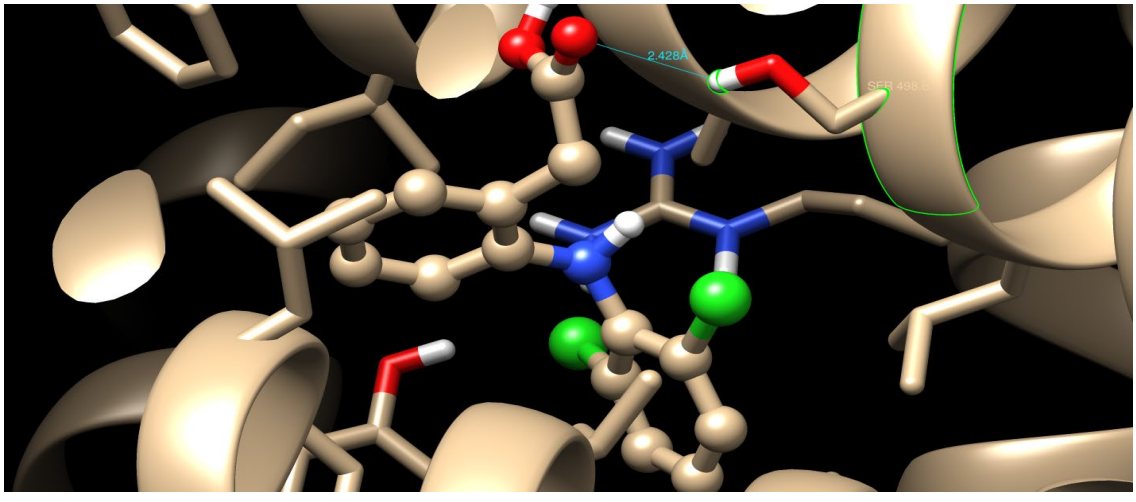

Figure 9. Interaction of diclofenac with prostaglandin G_1/H_1 synthase

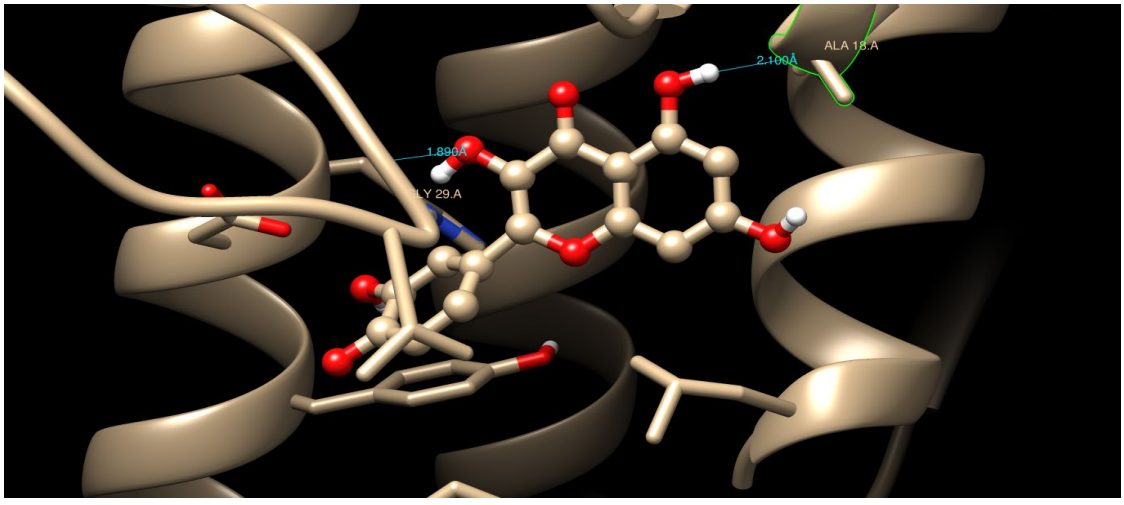

Figure 10. Interaction of quercetin with phospholipase A_2

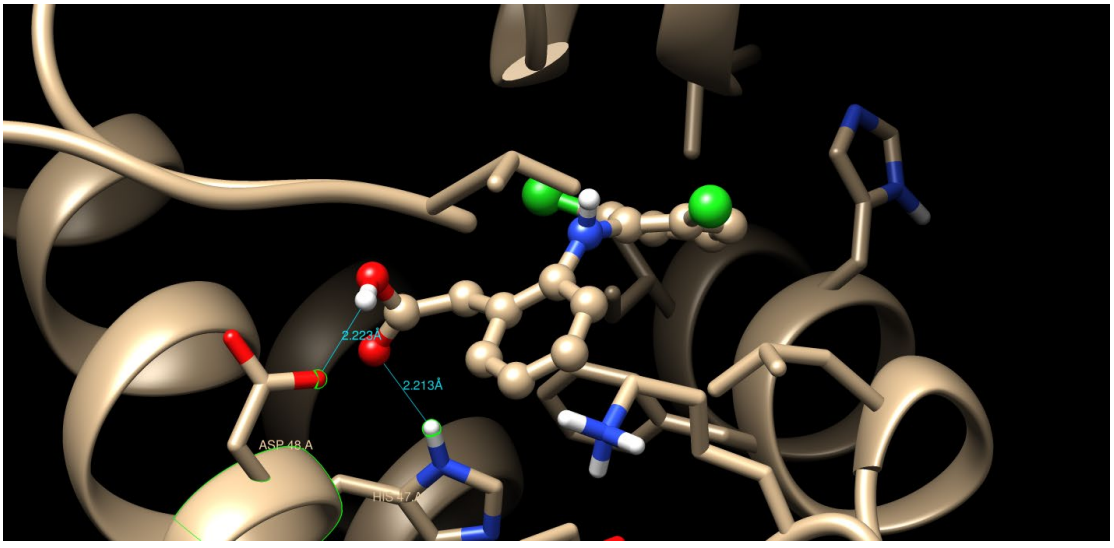

Figure 11. Interaction of diclofenac with phospholipase A_2

The results of ligand-protein binding, binding energy involved in amino acids and bond length of the ligand quercetin and standard antigout compound (allopurinol) and anti-inflammatory compound (diclofenac) with enzymes (xanthine oxidase, prostaglandin G_2/H_2, prostaglandin G_1/H_1 and phospholipase A_2) are given in Table 4.

In mcule software used in the study, few of the enzyme targets (animal origin) show docking sequence with ligand because in mcule, the gene sequence of a particular enzyme in humans is not studied completely, while studied in animals having biochemical and target sequence of animal enzymes showing resemblance to human biochemistry. Hence, to maintain consistency in the study using single software, mcule was used with different organisms enzymes targets available/studied. Moreover, animal enzymes are easily available to further pursue this research for wet-lab screening, sorting and optimizing the SAR. The animal enzymes considered for this study exhibit sufficient homology with human structure. Homology with human enzymes, resolution, availability, cost-effective approach and integrity of structure especially and other complicated errors related to software are considered vital.

Table 4. Molecular interaction of different enzymes with quercetin and standard compounds (Allopurinol and diclofenac)

Enzyme	Ligands	PDB ID	Organism	Amino acid	Binding energy (kcal/mol)	Bond length
Xanthine oxidase	Quercetin	2e1q	Homosapien	ALA 1054.D SER 1057.D	-9.2	2.280 Å 2.457 Å
	Allopurinol (standard inhibitor)	2e1q	Homosapien	GLY 772.D PHE 773.D GLN 1169.D GLN 1169.D	-6.4	2.054 Å 2.262 Å 2.265 Å 1.970 Å
Prostaglandin synthase G_2/H_2	Quercetin	3 mqe	Mus musculus	SER 499.C THR 175.C	-8.2	3.274 Å 3.118 Å
	Diclofenac (standard inhibitor)	3 mqe	Mus musculus	TYR 354.C SER 499.C	-8.0	2.451 Å 3.094 Å
Prostaglandin synthase G_1/H_1	Quercetin	1ht5	Ovis aries	ARG 88.B ARG 88.B	-7.4	1.917 Å 2.323 Å
	Diclofenac (standard inhibitor)	1ht5	Ovis aries	SER 498.B	-7.8	2.428 Å
Phospholipase A_2	Quercetin	Idb4	Homosapiens	GLY 29.A ALA 18.A	-8.0	1.890 Å 2.100 Å
	Diclofenac (standard inhibitor)	Idb4	Homosapiens	ASP 48A HIS 47.A	-7.7	2.223 Å 2.213 Å

The literature review indicated computational tools such as Metapocket and Autodock for finding molecular interaction of phospholipase A_2 with different phytochemicals (Shivashankar et al., 2019). Numerous studies reported that phenolic compounds and flavonoids inhibit eicosanoid biosynthesis, 5-lipoxygenase and cyclooxygenase pathways (Laughton et al., 1991; Ferrandiz and Alcaraz, 1991). Hence, they reduce the release of arachidonic acid and inhibit inflammation (Kim et al., 1998; Rathee et al., 2009).

CONCLUSION

The method developed in the present study was simple, reliable, sensitive, repeatable and reproducible, hence may be used to standardize polyherbal anti-gout remedy. The marker quercetin has shown good binding affinity with amino acid residues of xanthine oxidase, prostaglandin G_2/H_2 synthase, prostaglandin G_1/H_1 synthase and phospholipase A_2. The binding energies of quercetin against anti-inflammatory and anti-gout targets confirm the anti-gout activity of polyherbal medicine.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Development of method, experimentation, content writing and data analysis (SA), Materıals and processing the manuscript (HK, SA), Interpretation of results (HK, SA), Literature Search (HK, SA), Critical Reviews and final approval of manuscript (HK, SA).

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