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Research Articles

*Author of correspondence:

e-ISSN: 2687-6698 **Research Article**

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Investigation of acidic properties of 2-ethoxy-6-(3-substitue-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoates

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Abstract

In this study, to determine the pK_a values of 2-ethoxy-6-(3-substitue-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoates (**1-9**) were titrated potentiometrically with tetrabutylammonium hydroxide in four non-aqueous solvents such as isopropyl alcohol, *tert*-butyl alcohol, acetone and *N*,*N*-dimethylformamide (DMF), and graphs were drawn for all cases. In addition, the effects of solvents and molecular structure upon acidity compounds **1-9** were also determined and discussed. The half-neutralization potential values and the corresponding pK_a values were determined by the half neutralization method.

Keywords: 1,2,4-triazole, Schiff base, acidic properties, HNP, potentiometric titrations, pK_a

1. Introduction

Biological activities of 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives such as antifungal, antimicrobial, hypoglycemic, antihypertensive, analgesic, antiviral, anti-inflammatory, antitumor, antioxidant, and anti-HIV properties are well known [1-13]. Several reports explaining the synthesis of some *N*-arylidenamino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have been published [8-13].

Although water is an extraordinarily versatile solvent in which to carry out acid-base titrations, there are occasions when a nonaqueous solvent may be necessary or preferred, such as when the reagent is not water-soluble, and the neutralization reactions are not sufficiently complete in water. The completeness of a neutralization reaction depends, in part, on the acid or base strength of the analyte. But Bronsted and Lowry have made it clear that the observed acidity or basicity depends on the solvent because it is participating in the ionization [14]. There are two major factors such as solvent and structural effects influencing the acidity or basicity of a molecule. An acid or base too weak to titrate in water sometimes can be titrated in a non-aqueous solvent, where its observed acidity is greater [15-17].

Acidity measurements of organic compounds have carried out end of the 19th century when the first pK_a was measured. Since then, a large of data on the acidities of many solvents have been reported [18-21]. The measurements have mostly been made for polar solvents. Generally, water as a polar solvent has been used in these studies. Also, data have been reported for alcohols and dipolar aprotic solvents [22]. It is known that 1,2,4-triazole and 4,5-dihydro-1H-1,2,4-triazol-5-one rings possess weak acidic properties so that some 1,2,4-triazole and 4,5-dihydro-1H-1,2,4-triazol-5-one derivatives were titrated potentiometrically with tetrabutylammonium hydroxide in non-aqueous solvents. So, the pK_a values of the compounds were determined [9,10,17,22-29].

Determination of pK_a values of the active constituent of some pharmaceutical preparations is critical since the distribution, transport behavior, bonding to receptors, and contributions to the metabolic behavior of the active constituent molecules depend on the ionization constant [30-32].

The protonation constants of weak acidic compounds can be found by a number of different methods like potentiometric, chromatographic, electrophoretic methods [33]. In the present study, the pK_a values of some 1,2,4-triazole derivatives in non-aqueous media by using potentiometric measurements are found. These

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4,5-dihydro-1H-1,2,4-triazol-5-one derivatives have been prepared according to the reported reference in the literature [34].

2. Experimental

2.1. Chemistry

2-Ethoxy-6-(3-substitue-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoates (**1-9)** used in this study were prepared from the reactions of the corresponding 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones with 2-benzoxy-3-ethoxy-benzaldehyde as described in the literature [34]. Nine different 4,5-dihydro-1*H*-1,2,4-triazole derivatives [2-Ethoxy-6-(3-methyl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoate (**1**), 2-Ethoxy-6-(3-ethyl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoate (**2**), 2-Ethoxy-6-[3-(*n*-propyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine]-phenyl benzoate (**3**), 2-Ethoxy-6-(3-benzyl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoate (**4**), 2-Ethoxy-6-[3-(*p*-methylbenzyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine]-phenyl benzoate (**5**), 2-Ethoxy-6-[3-(*p*-methoxybenzyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine]-phenyl benzoate (**6**), 2-Ethoxy-6-[3-(*p*-chlorobenzyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine]-phenyl benzoate **(7)**, 2-Ethoxy-6-[3-(*m*-chlorobenzyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine]-phenyl benzoate (**8**) and 2-Ethoxy-6-(3-phenyl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl-azomethine)-phenyl benzoate (**9**)] were titrated with tetrabutylammonium hydroxide (TBAH) in four non-aqueous solvents (isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetone).

2.2. Potentiometric Titrations

For potentiometric titrations, an Orion 720A model pH ion meter equipped with a combined pH electrode (Ingold) and indicator electrode were employed. A 25 mL beaker, a magnetic stirrer, and a semi-micro burette were used in titrations. The pH meter was calibrated according to the instructions supplied by the manufacturers of the device before potentiometric titrations. In titrations, the titrant was added in increments of 0.05 mL then each stable reading and mV value were documented.

The used chemicals were supplied from Fluka and Merck. Purificated isopropyl alcohol was used to prepare a 0.05 N tetrabutylammonium hydroxide. 0.05 N tetrabutylammonium hydroxide in isopropyl alcohol was employed in potentiometric titrations. This solution was prepared from 0.1 N tetrabutylammonium hydroxide (TBAH) by dilution. The 0.05 M solution of TBAH in isopropyl alcohol as the titrant was used in the titration of acids. The half-neutralization potentials and the corresponding pK_a values of the compounds were determined from the potentiometric titrations with 0.05 M tetrabutylammonium hydroxide in isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide, and acetone. The mV values that were determined with the pH-meter.

The half neutralization potential (HNP) values and the found pK_a values of the compounds are given in Table **1**. The half-neutralization potential (HNP) values were calculated by drawing the mV-mL (TBAH) graphic. From the titration curves, the HNP values were found and the corresponding pK_a values were calculated [28,35].

3. Results and discussion

In this study, compounds **1-9** were titrated potentiometrically with TBAH in four non-aqueous solvents such as isopropyl alcohol (ε=17.9), *tert*-butyl alcohol (ε=12), acetone (ε=20.7) and *N*,*N*-dimethylformamide (ε=36.7). The mV values found in each titration were plotted against added 0.05 M TBAH volumes (mL) and potentiometric titration curves were obtained for all the experiments. From the titration curves, the HNP values were determined, and the corresponding pK_a values were founded.

The pH of weak acids can be calculated using Equation 1.

$$pH = pKa + log[A^-] / [HA] \qquad (1)$$

At the half-neutralization points, pH = pK_a when [A^-] is equal to [HA]. Therefore, the pH values at the half-neutralization points were stated as pK_a.

The half-neutralization potential (HNP) values and the corresponding pK_a values of compounds **1-9**, obtained from the potentiometric titrations with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, acetone, and DMF, are given in Table **1**.

As seen in Table **1**, for compounds **2** and **9** in *tert*-butyl alcohol, compound **5** in isopropyl alcohol and compound **8** in acetone, the HNP values and the corresponding pK_a values have not been obtained.

According to dielectric constant, the acidic arrangement can be expected as follows; *N*,*N*-dimethylformamide (ε=37) > acetone (ε=20.6) > isopropyl alcohol (ε=19.4) > *tert*-butyl alcohol (ε=12) [23-28,35]. However, as seen in Table **1**, the acidic arrangement for compounds **5** and **6** is: isopropyl alcohol > DMF > acetone > *tert*-butyl alcohol, for compound **1**, it is: acetone > DMF > *tert*-butyl alcohol, for compound **2**, it

is: isopropyl alcohol > acetone > DMF, for compound **3**, it is: DMF > isopropyl alcohol > *tert*-butyl alcohol > acetone, for compound **4**, it is: *tert*-butyl alcohol > DMF > isopropyl alcohol > acetone, for compound **7**, it is: acetone > isopropyl alcohol > *tert*-butyl alcohol > DMF, for compound **8**, it is: isopropyl alcohol > DMF > *tert*-butyl alcohol, and for compound **9**, it is: DMF > isopropyl alcohol > acetone.

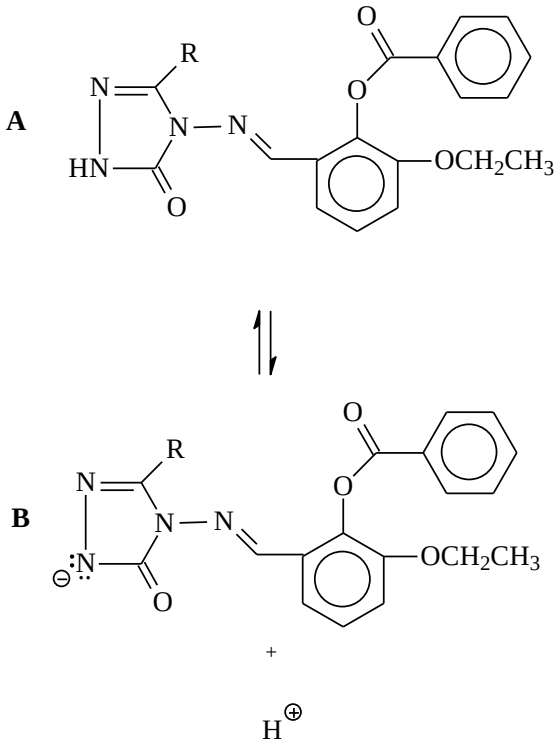
Table 1. The HNP and the corresponding pK_a values of compounds 1-9 in isopropyl alcohol, tert-butyl alcohol, DMF and acetone at 25 °C

Compound No	DMF		Acetone		tert-Butyl alcohol		Isopropyl alcohol	
	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a
1	-342	15.15	-316	14.12	-378	14.12	-	-
2	-361	15.66	-325	14.37	-365	15.05	-276	12.48
3	-172	11.08	-416	16.93	-340	14.32	-321	14.69
4	-372	15.88	-411	16.66	-193	8.14	-409	16.90
5	-267	13.35	-	-	-357	11.82	-211	11.93
6	-304	14.35	-446	17.30	-503	18.84	-	-
7	-324	14.85	-154	11.20	-229	12.10	-	-
8	-364	15.54	-	-	-409	16.22	-327	14.57
9	-267	13.22	-568	19.67	-	-	-393	16.07

In isopropyl alcohol, compounds **2**, **5**, **6**, and **8**, in acetone compounds **1** and **7**, in DMF compounds **3** and **9**, in *tert*-butyl alcohol, compound **4** show the strongest acidic properties. On the other hand, in *tert*-butyl alcohol, compounds **1**, **5**, **6**, and **8**, **in** acetone, compounds **3**, **4**, and **9**, **in** DMF, compounds **2** and **7**, show the weakest acidic properties. This situation may be attributed to the hydrogen bonding between the negative ions (**B**) formed and the solvent molecules in the amphiprotic neutral solvents [23,35-39] (Scheme **1**).

In addition, acidity is determined in each solvent and is observed in the following order: **7** > **5** > **6** > **2** > **3** > **8** > **9** > **4** in isopropyl alcohol, **4** > **7** > **3** > **5** > **1** > **8** > **6** **in** *tert*-butyl alcohol, **3** > **5** = **9** > **6** > **7** > **1** > **2** > **8** > **4** in DMF and **7** > **1** > **2** > **5** > **4** > **3** > **6** > **9** in acetone. According to these results, compound **7** in isopropyl alcohol and acetone, compound **4** in *tert*-butyl alcohol and compound **3** in DMF showed the strongest acidic properties; compound **4** in isopropyl alcohol and DMF, **6** in *tert*-butyl alcohol and compound **9** in acetone showed the weakest acidic properties [35].

The acidity of a compound alters according to some factors. The two most significant factors are solvent effects and molecular structure of the compound [9,10,12,15-17,22-27,36-40]. Table **1** shows that the HNP values and the corresponding pK_a values determined by the potentiometric titrations alter depending on the non-aqueous solvents in which the titration took place. Also, it is seen from Table **1** that the molecular structure of the compounds affects the HNP values as well as the corresponding pK_a values. Namely, the HNP values and corresponding pK_a values are related to the substituents linked to C-3 in 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring for the same solvent [40].



Scheme 1

The potentiometric titration curves of compounds **1-9** solutions titrated with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide, and acetone are given in Figure **1-9**. The mV values were plotted versus the added TBAH volumes (mL), and thus potentiometric titration curves were obtained for all the experiments. From these curves, the HNP values were determined and the corresponding pK_a values were calculated.

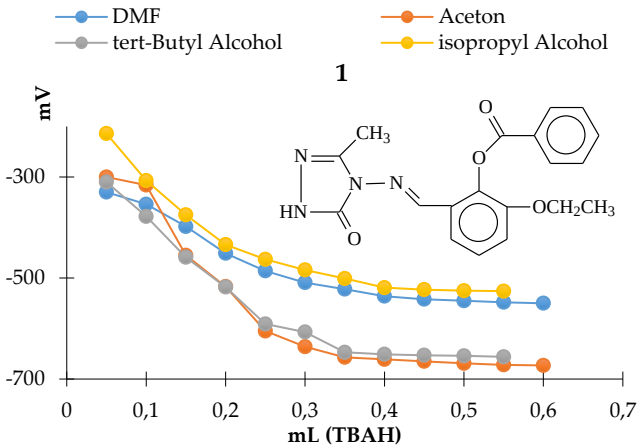


Figure 1. Potentiometric titration curves of 0.001 M solutions of compound **1** titrated with 0.05 M TBAH

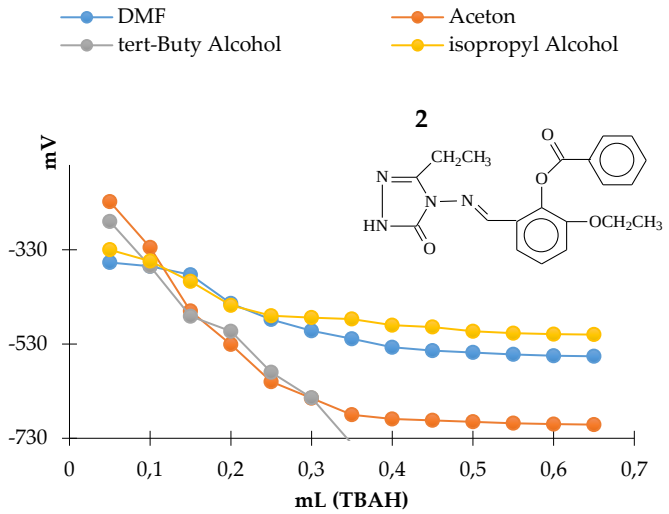


Figure 2. Potentiometric titration curves of 0.001 M solutions of compound **2** titrated with 0.05 M TBAH

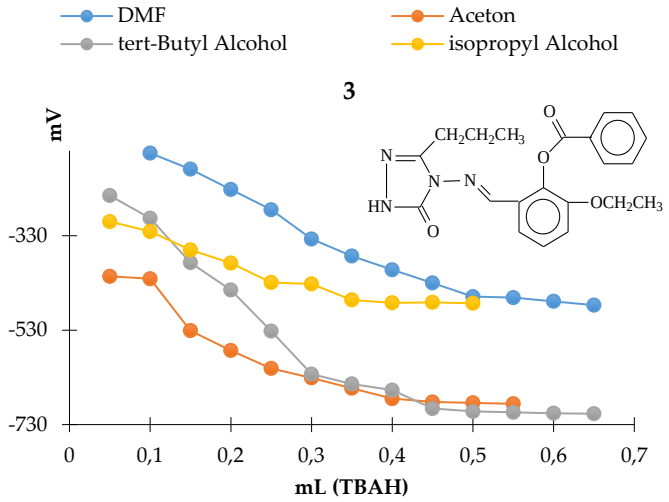


Figure 3. Potentiometric titration curves of 0.001 M solutions of compound **3** titrated with 0.05 M TBAH

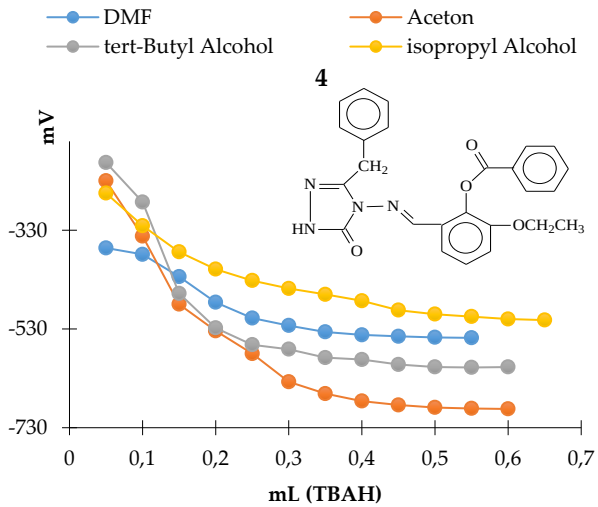


Figure 4. Potentiometric titration curves of 0.001 M solutions of compound **4** titrated with 0.05 M TBAH

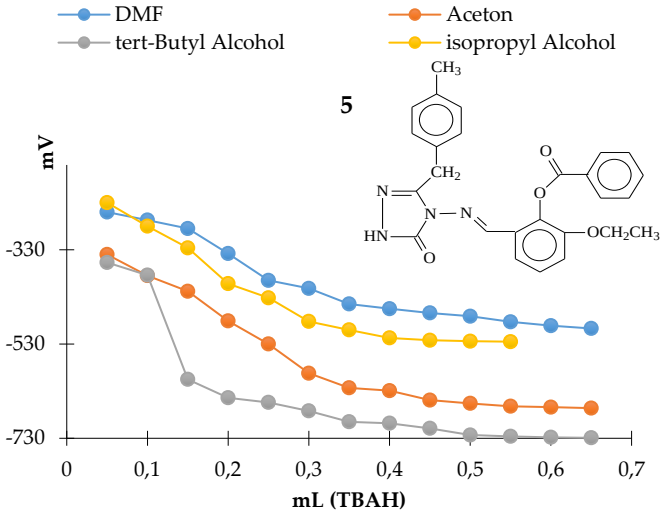


Figure 5. Potentiometric titration curves of 0.001 M solutions of compound **5** titrated with 0.05 M TBAH

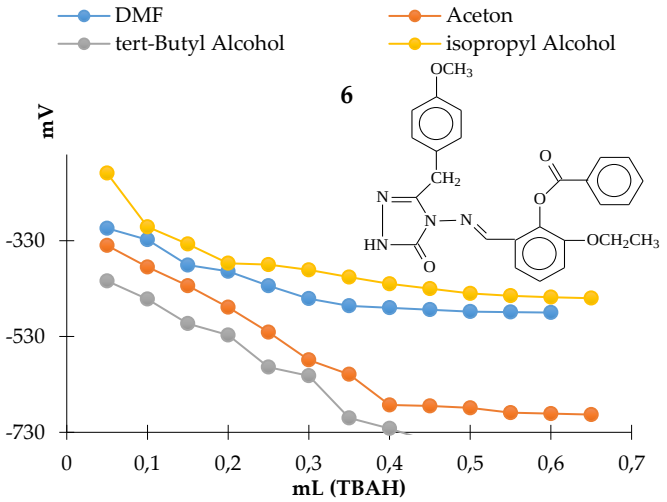


Figure 6. Potentiometric titration curves of 0.001 M solutions of compound **6** titrated with 0.05 M TBAH

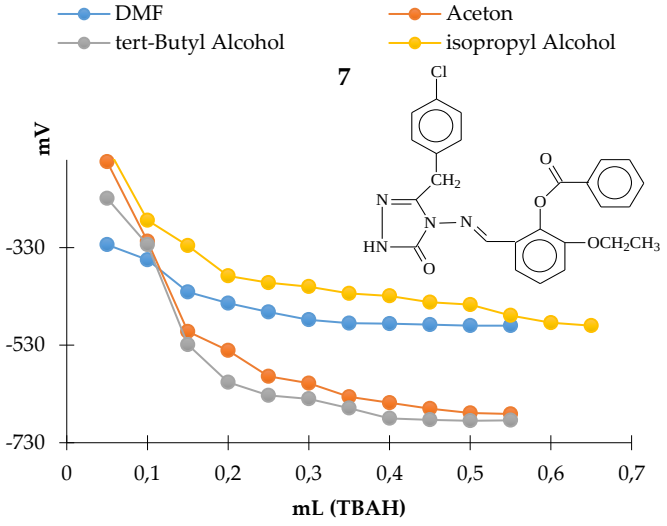


Figure 7. Potentiometric titration curves of 0.001 M solutions of compound 7 titrated with 0.05 M TBAH

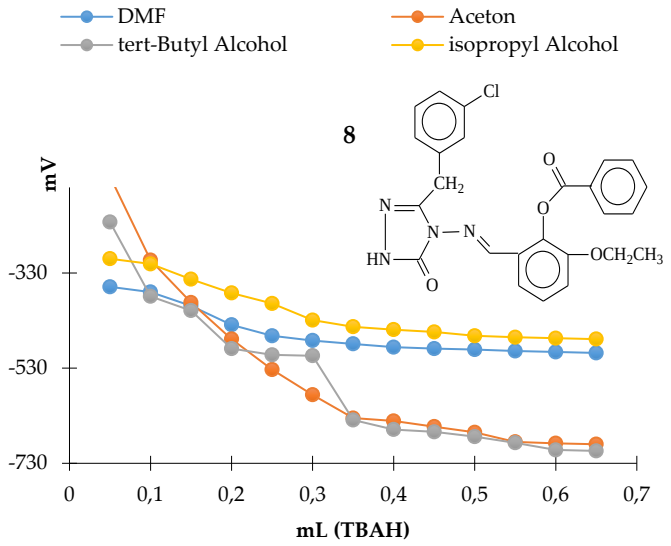


Figure 8. Potentiometric titration curves of 0.001 M solutions of compound **8** titrated with 0.05 M TBAH

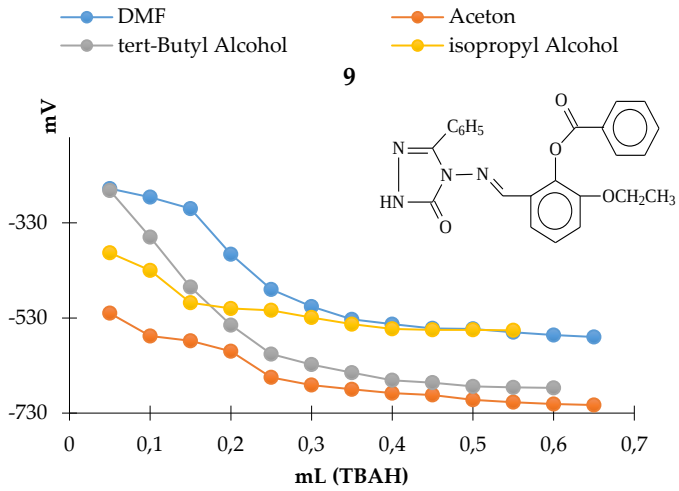


Figure 9. Potentiometric titration curves of 0.001 M solutions of compound **9** titrated with 0.05 M TBAH

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Designing potential HIV-1 integrase inhibitors: An *in silico* approach

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Abstract

Human immunodeficiency virus is a spectrum of conditions caused by infection with the human immunodeficiency virus. In 2019, about 38 million people worldwide were living with HIV and 690,000 deaths had occurred in that year. To date, for the treatment of HIV-1 disease, many compounds have been synthesized and some of them were approved by FDA. However, the use of these drugs has been limited due to reasons such as resistance caused by the misuse of drugs and bad side effects. We describe herein designing 48 novel compounds as a potential inhibitor of HIV-1 integrase through *in silico* studies such as molecular docking, target analysis, toxicity prediction, and ADME prediction. The online web-based platform, SwissADME, also predicts these molecules' solubility, pharmacodynamics property, and target accuracy.

Keywords: Swissadme, HIV-1 integrase, molecular docking, *in silico* study, toxicity

1. Introduction

HIV-1 integrase (IN) represents a charming aim in anti-HIV drug design fundamentally because of its specificity. Hence, HIV-1 IN does not possess a functional equivalent in humans and exhibits a crucial role in forming irreversible and productive viral infections [1]. This enzyme catalyzes the insertion of proviral DNA, obtained from reverse transcription of HIV-1 RNA, into the genome of the host-infected cells. The insertion is carried through a two-step enzymatic phase which is endonucleolytic cleavage of a terminal dinucleotide (GT) and "strand transfer" (ST). Consequently, both reactions are finalized by the catalytic core domain of HIV-1 IN which includes two divalent metal ion cofactors (Mg^{2+}). These metal ions are coordinated by three catalytic carboxylate residues: Asp64, Asp116, and Glu152 (DDE triad) within the enzyme active site [2]. To aim the metal cofactors within the active site of a viral metal-activated enzyme-like HIV-1 IN has shown up as an appealing and confirmed strategy for the improvement of novel anti-HIV agents. For this purpose, a metal-binding pharmacophore model has been utilized to design novel HIV-1 IN inhibitors as given in Fig. 1A. Two distinctive features are particularly taken into account in this model: the first is a planar metal binding site that can interact with metals present in the enzyme's active site, while the second is the presence of an aromatic or heteroaromatic hydrophobic functional group located close to the metal-binding site [3,4]. Constant workings in using this pharmacophore model have ended up the design and following FDA approval of three INIs for clinical use as potent anti-HIV drugs: Raltegravir (RLT), Elvitegravir (EVG), and Dolutegravir (DTG) in 2007, 2012, and 2013, respectively (Fig. 1B) [5-7].

Various MBGs have been widely researched to design progressive and potent INIs [8,9]. Isonaphthylic acid (INA) derivatives can potentially be studied as novel HIV-1 IN inhibitors due to the metal-binding sites in their structure. Thus, INA derivatives represent an effective class of aromatic ligands with powerful bidentate chelating capacity toward metal ions. Moreover, the presence of an amide group as a linker plays an important role in the inhibitory activity of these types of compounds. And an aromatic functional group linked to the amide is considered for ensuring the basic interactions with the hydrophobic pocket of the enzyme [10].

In drug discovery studies, many parameters such as which reactants to be used in which amount, substituent selection, and whether they are biologically active or not are evaluated in order to avoid time and resource

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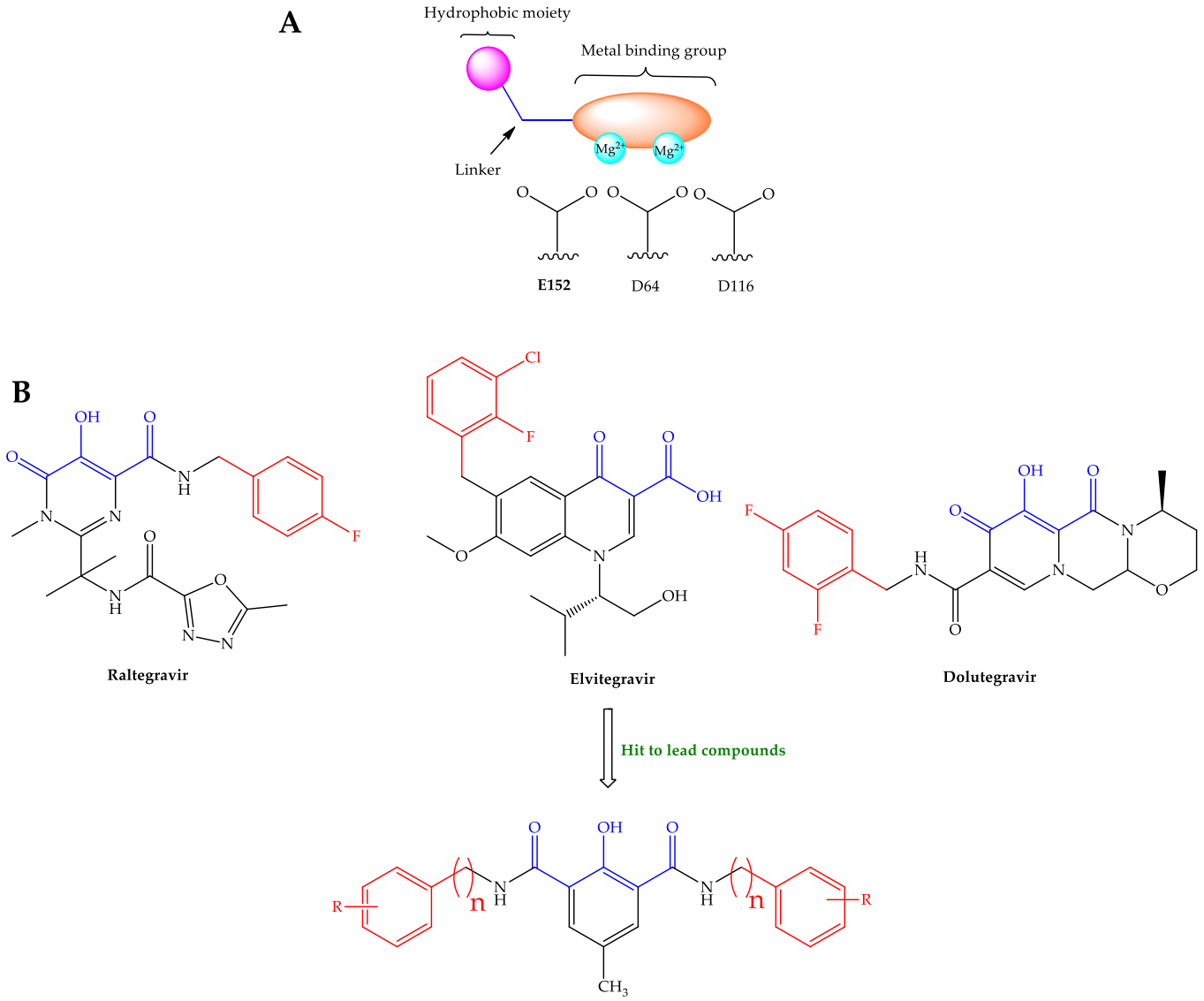


Figure 1. Demonstration of the pharmacophore model for HIV-1 INs. (**B**) FDA-approved drugs against HIV-1 INIs. Atoms marked with blue of MBG of the drugs can chelate the Mg^{2+} ions. The hydrophobic part of the compounds is marked with red

consumption. The molecule to be synthesized must have high activity and low toxicity at the same time. Equally significant is the access to and concentration at the therapeutic target in the organism. The conventional step to consider pharmacokinetics is to separate the diverse impacts that influence the access to the target into individual parameters. In this context, these ADMET parameters (for absorption, distribution, metabolism, excretion, and toxicity) can be evaluated separately by dedicated methods. In drug design and discovery studies, it has been dedicated that the early estimation of ADMET properties greatly reduces the potential disadvantages in clinical and phase stages. Computer models have been encouraged as an effective alternative to experimental procedures for the estimation of ADME, particularly at early steps, when researched chemical structures are countless but the availability of compounds is limited [11,12].

The SwissADME is a web-based platform that is freely accessible at http://www.swissadme.ch and it can be simply used even by non-experts in computer-aided drug design (CADD) studies and the results can be easily analyzed. Compared to other web-based tools for determining ADME and pharmacokinetic properties, SwissADME key points are, partially: various input ways, computation for multiple compounds, and the opportunity to show, save and share outcomes per individual compounds or through global intuitive and interactive graphs. Consequently, SwissADME is combined with the SwissDrugDesign field. One-click interoperability gains access to different CADD tools improved by the Molecular Modeling Group of the SIB Swiss Institute of Bioinformatics, e.g., ligand-based virtual screening, bio target prediction, molecular docking, bioisosteric design, or molecular mechanics [13-21].

In the light of the above considerations, this paper includes *in silico* analysis of 48 novel HIV-1 IN inhibitors, containing different substituents and linkers such as fluorine, chlorine, bromide, methoxy, which is provided

with the target protein's inhibition site (PDB ID: 1QS4), prediction of ADME, Target Prediction were done by using SwissBioinformatics online Tools. The prediction of toxicity of designing compounds was screened via the pkCSM online web tool. Furthermore, the molecular docking studies of the most potent compound were performed.

2. Materials and Methods

2.1. Molecular Docking

The co-crystallized structure of HIV-1 integrase enzyme [PDB: 1QS4] was taken from Protein Data Bank (PDB) and prepared by utilizing the Protein Preparation Wizard Module of Schrödinger Software Suite. Then it was optimized by removing the water molecules, heteroatoms, and co-factors. The hydrogens, missing atoms, bonds, and charges were computed through Maestro [26, 27]. Compound **24** exhibited the highest drug-likeness score was selected for docking study. The ligand preparation and optimization contain forming different tautomers, assigning bond orders, ring conformations that were minimized using the OPLS2005 force field before the docking study, and stereochemistries were performed via the LigPrep module of Schrödinger Software Suite. Besides, a receptor grid was formed around the co-crystallized ligand of the enzyme. The grid box size was set to 20 Å Radius, using the receptor Grid Generation applied in Glide. Extra Precision (XP) mode and Glide programs were used for the docking calculations [28-30].

2.2. ADME Prediction

ADME (adsorption, distribution, metabolism, and excretion) is significant to search the pharmacodynamics of the designed compounds which could be a target agent in drug design and discovery studies. SwissADME is a web-based platform that lets the user upload or draw their hit compounds with structure or SMILES code. This tool supplies many parameters such as lipophilicity (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT, Log $P_{o/w}$), water solubility- Log S (ESOL, Ali, SILICOS-IT), drug-likeness rules (Lipinski, Ghose, Veber, Egan, and Muegge) and Medicinal Chemistry (PAINS, Brenk, Leadlikeness, Synthetic accessibility) methods [22]. The designed novel HIV-1 IN inhibitors were uploaded with SMILES codes and analyzed.

2.1. Target Prediction

Molecular Target investigations are crucial to determine the phenotypical side effects or potential cross-reactivity induced by the action of bioorganic compounds of which molecular weight is not bigger than 500 g/mol [23]. The designed compound's SMILES codes were uploaded to the Swiss Target Prediction website in order to analyze their target prediction (https://www.swisstargetprediction.ch).

2.2. Toxicity Prediction

Toxicology prediction of bioorganic compounds is substantial to estimate the amount of tolerability of the hit compounds before *in vitro*, *in vivo*, and clinical studies. pkCSM is also a web-based platform for analyzing physicochemical properties of small compounds, and this online website supplies many toxicology results such as LD50, hERG-I inhibitor, AMES Toxicity, hERG-II inhibitor, human maximum tolerated dose, LOAEL, Skin Toxicity, *T. pyriformis* toxicity, Hepatotoxicity, and Minnow toxicity. The designed compounds' SMILES codes were uploaded pkCSM website to analyze their target prediction (http://biosig.unimelb.edu.au/pkcsm/) [24].

3. Results and Discussion

3.1. Molecular Docking

Considering the ADME and toxicity outcomes, a deep docking study was implemented to regard the possible binding modes of the promising compound (24) inside the active site of HIV-1 integrase enzyme (PDB ID: 1QS4) by using Schrödinger Software. Initially, the co-crystalized ligand 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone (CCL) and the protein (PDB ID: 1QS4) were modeled to validate the Glide. Superimposition of the experimental bound (co-crystallized) conformation of 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone estimated by Glide. The experimental binding conformations of co-crystalized ligand in the binding pocket of 1QS4 were successfully generated with an acceptable root-mean-square deviation (RMSD) of 0.175 Å (< 2 Å) [31]. The interactions between co-crystallized ligand and the active site of HIV-1 IN was shown in Fig. 2A. Compound **24** and its interactions with the binding side of 1QS4 were demonstrated in Fig. 2B. Hereby, compound **24** was surrounded with LYS 159, LYS 156, ASN 155, GLU 152, ILE 151, GLY 149, and GLN 148 amino acid residues of the active site of HIV-1 IN enzyme. Further, the carbonyl group of amide generated a strong hydrogen-bonding interaction with ASN 155, and the hydroxy group between two amide bonds in the phenyl ring formed a strong hydrogen-bonding interaction with ASP 64. The docking score of the chosen compound and co-crystallized ligand were given in Table 1.

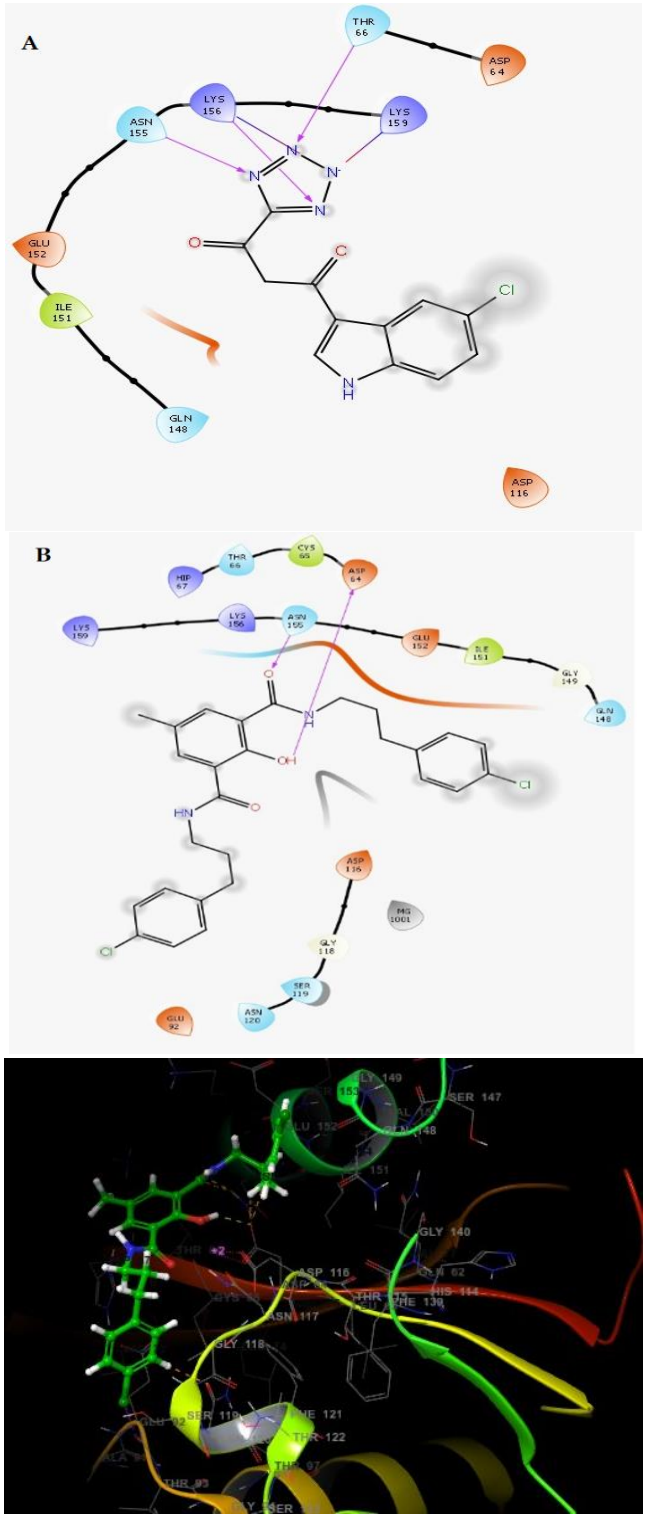


Figure 2. **A**: Co-crystallized ligand and its interaction of active site of HIV-1 IN, **B**: Compound **24** and its interaction of active site of HIV-1 IN

Table 1. The docking scores the chosen compound, co-crystallized ligand and its binding interactions with the active site of HIV-1 IN (PDB ID: 1QS4)

Ligand	Docking score	H-bond	Metal coordination	Pi-Pi stacking	Pi-cation	Salt bridge
CCL	-7.5	ASN 155 LYS 156 THR 66 LYS 159	x	x	x	x
24	-6.890	ASN 155 ASP 64	x	x	x	x

Table 2. The physiochemical property results of the designed compounds

Comp. No	TPSA (Å²)	*n*HD	*n*HA	Log $P_{o/w}$	Log *S*	Log *Kp* (cm/s)	Lipinski	Druglikeness Score
1	78.43	3	5	4.13	-5.03	-5.54	0	0.24
2	78.43	3	5	4.16	-4.97	-5.8	0	0.25
3	78.43	3	5	4.70	-5.56	-5.32	1	0.31
4	78.43	3	5	5.38	-6.02	-4.99	1	0.35
5	78.43	3	5	4.01	-5.03	-5.54	0	0.11
6	78.43	3	5	4.24	-4.97	5.8	0	0.49
7	78.43	3	5	4.80	-5.56	-5.32	1	0.58
8	78.43	3	5	5.35	-6.02	-4.99	1	0.63
9	78.43	3	5	3.99	-5.03	-5.54	0	0.60
10	78.43	3	5	4.17	-4.97	-5.8	0	0.54
11	78.43	3	5	4.72	-5.56	-5.32	1	0.66
12	78.43	3	5	5.40	-6.02	-4.99	1	0.72
13	78.43	3	3	4.49	-5.9	-4.99	0	0.42
14	78.43	3	3	4.68	-5.83	-5.26	0	0.46
15	78.43	3	3	5.17	-6.43	-4.77	1	0.50
16	78.43	3	3	5.76	-6.9	-4.43	1	0.53
17	78.43	3	3	4.46	-5.9	-4.99	0	0.18
18	78.43	3	3	4.55	-5.83	-5.26	0	0.58
19	78.43	3	3	5.12	-6.43	-4.77	1	0.66
20	78.43	3	3	5.68	-6.9	-4.43	1	0.71
21	78.43	3	3	4.45	-5.9	-4.99	0	0.77
22	78.43	3	3	4.68	-5.83	-5.26	0	0.71
23	78.43	3	3	5.20	-6.43	-4.77	1	0.81
24	78.43	3	3	5.85	-6.9	-4.43	1	0.86
25	78.43	3	3	4.69	-6.54	-5.44	2	0.16
26	78.43	3	3	4.81	-6.46	-5.71	2	0.19
27	78.43	3	3	5.34	-7.06	-5.22	2	0.25
28	78.43	3	3	5.90	-7.53	-4.88	2	0.25
29	78.43	3	3	4.61	-6.54	-5.44	2	-0.02
30	78.43	3	3	4.74	-6.46	-5.71	2	0.39
31	78.43	3	3	5.31	-7.06	-5.22	2	0.47
32	78.43	3	3	5.93	-7.53	-4.88	2	0.52
33	78.43	3	3	4.61	-6.54	-5.44	2	0.46
34	78.43	3	3	4.78	-6.46	-5.71	2	0.42
35	78.43	3	3	5.41	-7.06	-5.22	2	0.53
36	78.43	3	3	5.94	-7.53	-4.88	2	0.59
37	96.89	3	5	3.57	-5.21	-5.48	0	-0.18
38	96.89	3	5	3.50	-4.79	-6.13	0	0.12
39	96.89	3	5	4.16	-5.39	-5.65	0	0.20
40	96.89	3	5	4.70	-5.86	-5.31	0	0.25
41	96.89	3	5	3.38	-4.86	-5.87	0	-0.04
42	96.89	3	5	3.50	-4.79	-6.13	0	0.39
43	96.89	3	5	4.17	-5.39	-5.65	0	0.54
44	96.89	3	5	4.72	-5.86	-5.31	0	0.60
45	96.89	3	5	3.41	-4.86	-5.87	0	0.30
46	96.89	3	5	3.52	-4.79	-6.13	0	0.32
47	96.89	3	5	4.17	-5.39	-5.65	0	0.48
48	96.89	3	5	4.72	-5.86	-5.31	0	0.55

Comp.: Compound

3.2. ADME Prediction

The ADME predictions of the designed compounds were performed by SwissADME database, and

physiochemical properties; hydrogen bond acceptors (*n*HA), hydrogen bond donors (*n*HD), and topological polar surface area (TPSA). Besides, lipophilicity (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT, and Consensus $P_{o/w}$), water-solubility properties (ESOL, Ali, SILICOS-IT), and drug-likeness factors (Lipinski's Rules, Ghose, Veber, Egan, Muegge) were also calculated. All designed compounds showed high topological polar surface area (TPSA) ranging from 78.43 Å^2 to 96.89 Å^2. The methoxy-containing compounds were found to be higher than the others. *n*HD of the compounds was found 3, whereas *n*HA of compounds was between 3 and 5. The calculated lipophilicity properties were given in the consensus model (*n*-octanol and water: Log $P_{o/w}$) which was ranging from 3.41 to 5.94. When the water solubility properties were examined, all compounds were found to be moderately or poorly soluble with the Log *S* values ranging from -4.79 to -7.53 mg/mL. Pharmacokinetic properties such as GI absorption, BBB permeant, P-gp substrate, and skin permeation (Log *K*p) were also calculated, and the designed compounds exhibited high Gastrointestinal absorption (GI), while none of them showed blood-brain barrier permeant. Skin permeation kinetics (Log *K*p) of them were found to be -4.43-6.13 cm/s (Table 2).

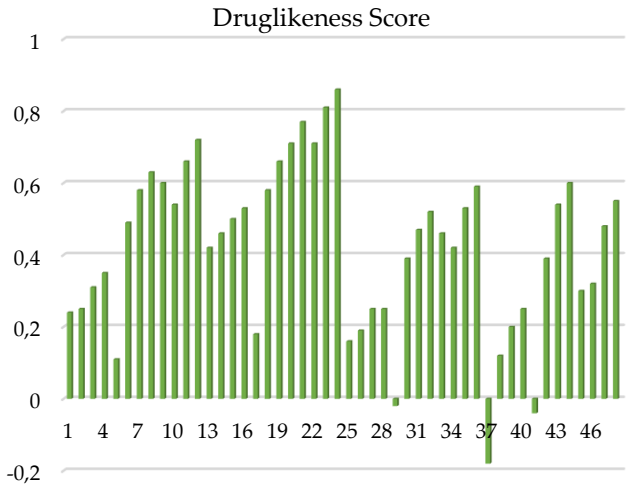


Figure 3. Drug-likeness score of the designed compounds

The Lipinski rule of five is an important parameter for drug-likeness factors of the compounds, and it has to be ≤ 1. Among the 48 designed compounds, compounds **25**-**36** containing bromophenyl as a hydrophobic moiety did not obey this rule, and for this reason, these compounds were eliminated. Drug-likeness scores was specified via the Molsoft database (www.molsoft.com). Although all compounds showed drug-likeness score with varying values, among them, compounds **12**, **21**-**24** displayed the highest values with 0.72, 0.77, 0.71, 0.81, and 0.86, respectively.

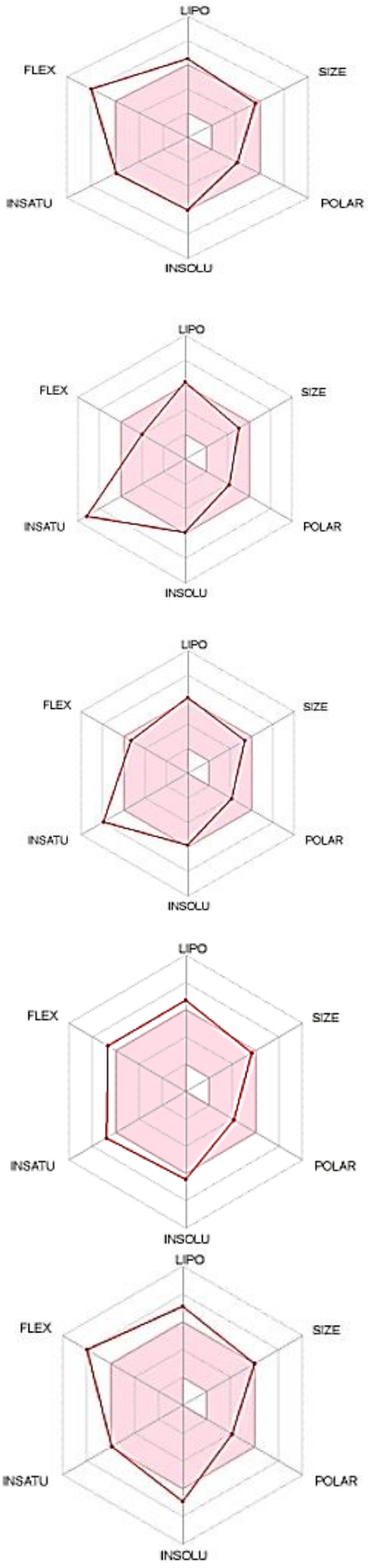


Figure 4. The Bioavailability Radar enables at first glance at the drug-likeness of the compounds

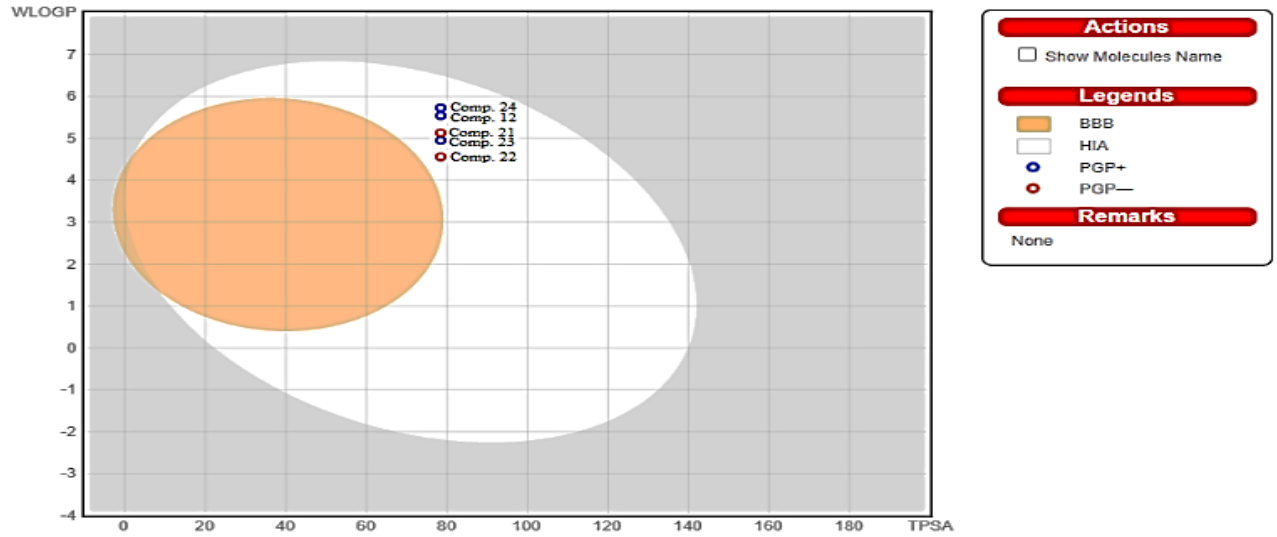


Figure 5. BOILED-Egg presentation of the compounds

Compared to methoxy-containing compounds which showed the highest TPSA results with 96.89, these five compounds exhibited a 1-6-fold better drug-likeness score than them (All SwissADME results were given in detail in Fig. 3). Moreover, the bioavailability score of these compounds was found to be 0.55 (Fig. 4). For further *in silico* analysis, the above compounds were chosen as hit compounds.

In Fig. 4, the pink area describes the optimal range for each property (lipophilicity: XLOGP3 between -0.7 and +5.0, size: MW between 150 and 500 g/mol, polarity: TPSA between 20 and 130 Å^2, solubility: log *S* not higher than 6, saturation: fraction of carbons in the sp^3 hybridization not less than 0.25, and flexibility: no more than 9 rotatable bonds.

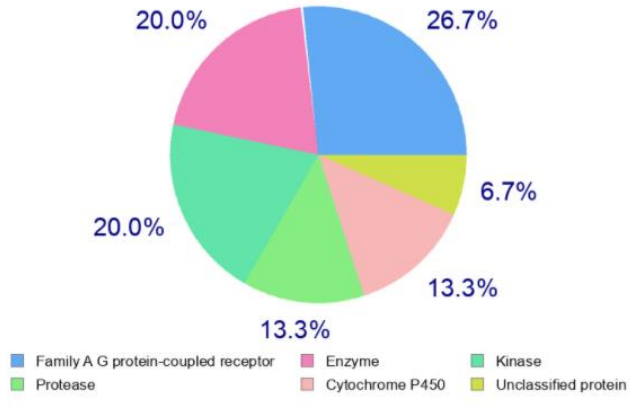
Furthermore, the BOILED-Egg profile which lets for intuitive consideration of passive gastrointestinal absorption (HIA) and brain penetration (BBB) in the function of the position of the molecules in the WLOGP-vs-TPSA referential was screened for the selected five compounds [25]. The white area is for the high probability of passive absorption by the gastrointestinal tract, while the yellow area is for the high probability of brain penetration. Also, the marks are colored in blue if predicted as actively effluxed by P-gp (PGP+) and in red if estimated as non-substrate of P-gp (PGP-). It was concluded that all our compounds were estimated well-absorbed but not accessing the brain, and compounds **12**, **23**, and **24** were subject to active efflux (blue dot), whereas compounds **21** and **22** were not subject to active efflux (red dot) (Fig. 5).

3.3. Target Prediction

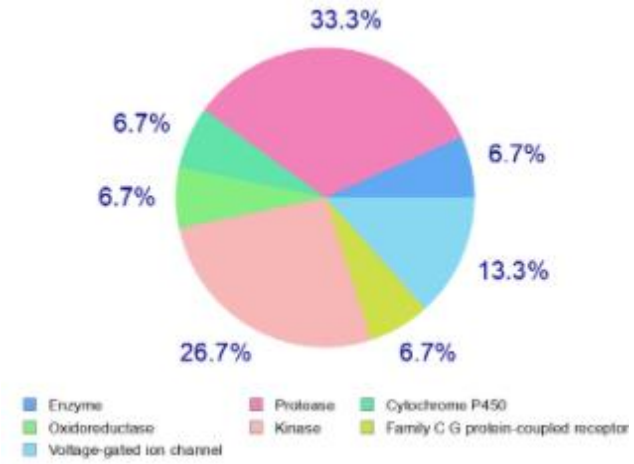
The target estimation of the chosen compounds was examined using the SwissTargetPrediction platform with the following investigations 15 of the outcomes depicted as a pie-chart (Fig. 6). The compound 12 containing *p*-fluorophenyl as a hydrophobic aromatic unit and n = 3 as a linker was predicted as 26.7% of Family AG protein-coupled receptor, 20% of both enzyme and kinase, while compound 21 comprising *p*-chlorophenyl as a hydrophobic aromatic unit and n = 0 was predicted as 33.3% of protease and 26.7% of the kinase. Compounds 22 and 23 were estimated as Family AG protein coupled-receptor with 40.0% and 46.7%, respectively, whereas compound 24 including *p*-chlorophenyl as a hydrophobic aromatic unit and n = 3 was predicted as 46.7% of Family AG protein coupled-receptor and 20% of the enzyme. One of the compound's result tables comprising of Common Name, Uniprot ID, Target, ChEMBL-ID, Probability, Target Class, and Known actives in 2D/3D are given in the Supporting Information file.

3.4. Toxicity Prediction

Toxicity predictions were screened and all compounds, except for **22**, do not have any AMES toxicity, while all compounds, except for **12**, produce hepatotoxicity. Moreover, all compounds were not found to be cause skin sensitivity. Although they were also predicted as hEGR II inhibitors, none of them showed any hEGR I inhibition results. It was estimated maximum tolerated dose for humans which was ranging from 0.426 log mg/kg/day to 0.79 log mg/kg/day. Compound **12** exhibited the lowest both oral rat acute toxicity with a LD_{50} value of 2.271 mol/kg and chronic oral rat toxicity with 1.497 log mg/kg_bw/day. 0.397 - 0.672 log mg/L induced *T. Pyriformis* toxicity and -0.765 - 0.027 log mM induced Minnow toxicity were found for the compounds (Table 3).



Compound 12



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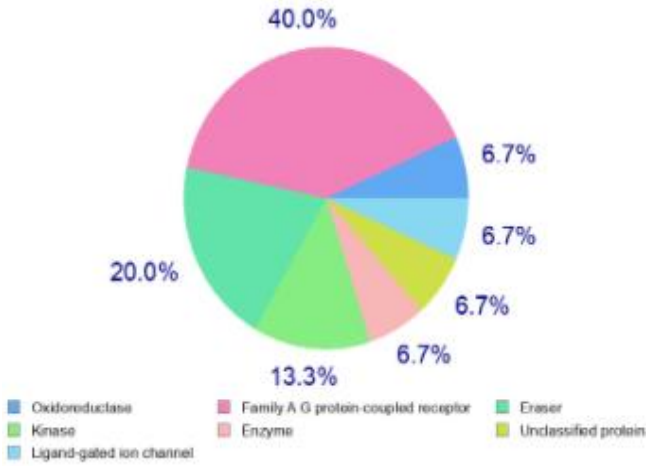
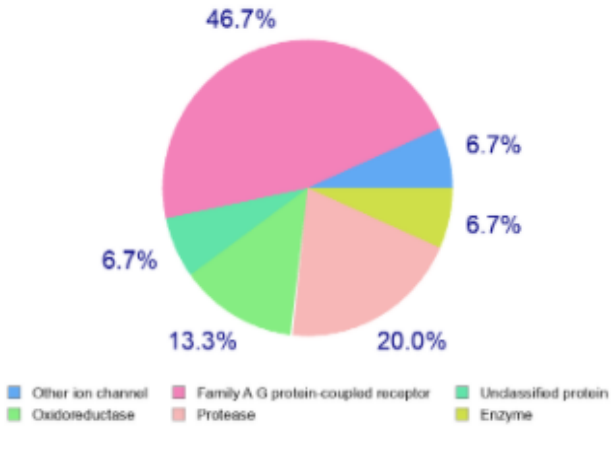
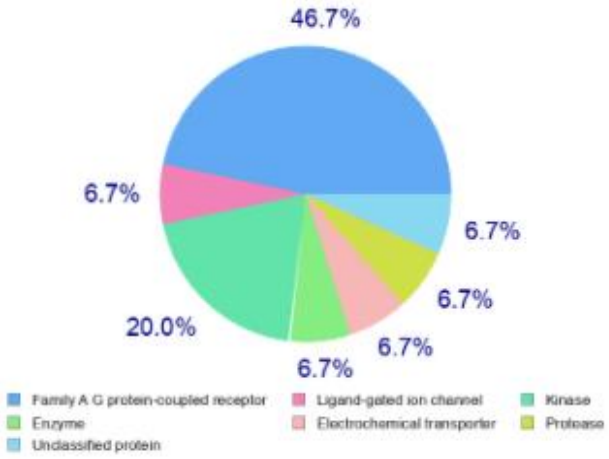


Compound 22



Compound 23



Compound 24

Figure 6. SwissTargetPrediction of the chosen compounds

Table 3. Toxicity prediction results of the selected compounds

Comp. No	AMES Toxicity	Maximum Tolerated Dose	hEGR I inhibitor	hEGR II inhibitor	Oral Rat Acute Toxicity (LD_{50})	Chronic Oral Rat Toxicity (LOAEL)	Hepatotoxicity	Skin Sensitisation	*T.Pyriformis* Toxicity	Minnow Toxicity
12	No	0.457	No	Yes	2.271	1.497	No	No	0.397	-0.954
21	No	0.426	No	Yes	2.462	1.757	Yes	No	0.464	-1.005
22	Yes	0.790	No	Yes	2.395	1.536	Yes	No	0.470	-0.765
23	No	0.581	No	Yes	2.455	1.631	Yes	No	0.672	0.027
24	No	0.520	No	Yes	2.414	1.740	Yes	No	0.555	-0.779

Comp.: Compound

4. Conclusion

In the present study, we have investigated the novel potential compounds against HIV-1 IN with the *in silico* studies. We designed 48 novel compounds with various functional groups such as -F, Cl, -Br, -OCH3 in the phenyl ring which is the hydrophobic site, and we added to the structure linker which is the alkyl chain between amide bond and hydrophobic part. It was observed that the presence of carbonyl and hydroxyl group in the compound was very significant for the binding of the active site of the enzyme. Also, it can be concluded from the SwissADME and toxicity results that the alkyl chain length was also important for both solubility and drug-likeness. It was determined that the solubility decreases as the alkyl chain length increases in the compounds, on the contrary, it was found that there is an increase in the drug-likeness score as the alkyl number increases. We also considered the position of the substituent in the phenyl ring for the activity when designing the compounds, and it was clearly seen that the substituent in the p-position was more important than the other positions. The bromine compounds were found to be more potential for activity, whereas methoxy-containing compounds had less potential. For the best compound, we performed molecular docking studies and hydrogen bonding interactions between ligands and ASN155, ASP 64 amino acids of the receptor exhibited significance for potent HIV-1 IN. Considering the overall results, the five compounds and especially compound **24** can be a potent inhibitor against HIV-1 IN.

Declaration of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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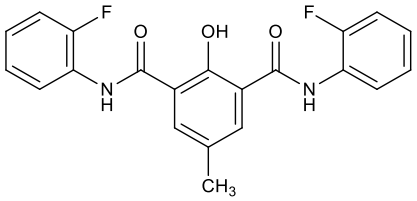
Supporting information

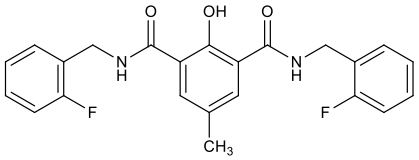
SwissTargetPrediction

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Dopamine D2 receptor (by homology)	DRD2	P14416	CHEMBL217	Family A G protein-coupled receptor	0.120225750913	0 / 405
Serotonin 2a (5-HT2a) receptor (by homology)	HTR2A	P28223	CHEMBL224	Family A G protein-coupled receptor	0.120225750913	0 / 140
11-beta-hydroxysteroid dehydrogenase 1	HSD11B1	P28845	CHEMBL4235	Enzyme	0.120225750913	0 / 150
MAP kinase p38 alpha	MAPK14	Q16539	CHEMBL260	Kinase	0.120225750913	0 / 37
Serine/threonine-protein kinase Chk1	CHEK1	O14757	CHEMBL4630	Kinase	0.120225750913	1 / 0
Serine/threonine-protein kinase WEE1	WEE1	P30291	CHEMBL5491	Kinase	0.120225750913	4 / 0
Trypsin I	PRSS1	P07477	CHEMBL209	Protease	0.120225750913	0 / 28
Epoxide hydratase	EPHX2	P34913	CHEMBL2409	Protease	0.120225750913	0 / 57
Poly [ADP-ribose] polymerase-1	PARP1	P09874	CHEMBL3105	Enzyme	0.120225750913	0 / 84
Cytochrome P450 2C9	CYP2C9	P11712	CHEMBL3397	Cytochrome P450	0.120225750913	0 / 7
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0.120225750913	0 / 7
Poly [ADP-ribose] polymerase 2	PARP2	Q9UGN5	CHEMBL5366	Enzyme	0.120225750913	0 / 22
Calcitonin gene-related peptide 1	CALCA	P06881	CHEMBL5293	Unclassified protein	0.120225750913	6 / 0
C-C chemokine receptor type 4	CCR4	P51679	CHEMBL2414	Family A G protein-coupled receptor	0.0	2 / 0
Interleukin-8 receptor A	CXCR1	P25024	CHEMBL4029	Family A G protein-coupled receptor	0.0	12 / 0
C-C chemokine receptor type 6	CCR6	P51684	CHEMBL4423	Family A G protein-coupled receptor	0.0	2 / 0
CCR4-NOT transcription complex subunit 7	CNOT7	Q9UIV1	CHEMBL3616361	Hydrolase	0.0	1 / 0
Glycine transporter 2	SLC6A5	Q9Y345	CHEMBL3060	Electrochemical transporter	0.0	0 / 41
Mu opioid receptor	OPRM1	P35372	CHEMBL233	Family A G protein-coupled receptor	0.0	0 / 411
Kappa Opioid receptor	OPRK1	P41145	CHEMBL237	Family A G protein-coupled receptor	0.0	0 / 219
Delta opioid receptor (by homology)	OPRD1	P41143	CHEMBL236	Family A G protein-coupled receptor	0.0	0 / 349
Plasminogen	PLG	P00747	CHEMBL1801	Protease	0.0	0 / 3
C-C chemokine receptor type 3	CCR3	P51677	CHEMBL3473	Family A G protein-coupled	0.0	0 / 21

SI-1. Target Prediction results of the compound 12



Compound 1



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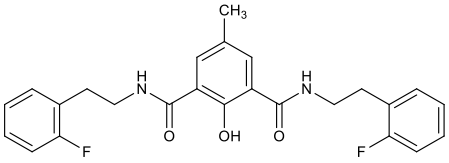

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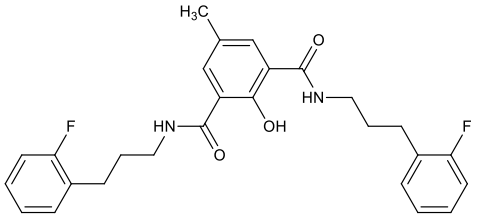

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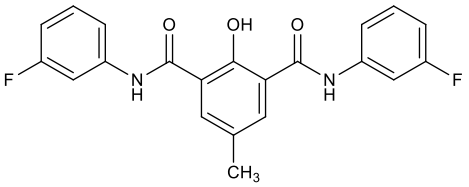

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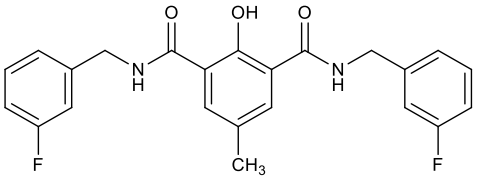

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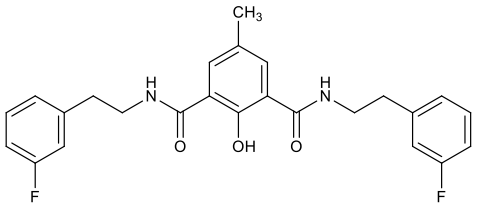

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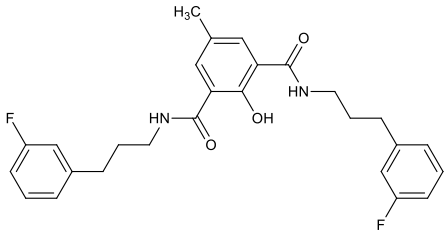

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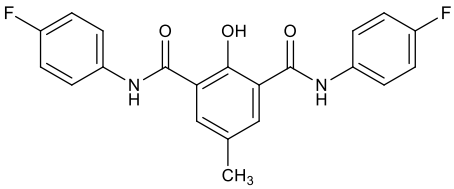

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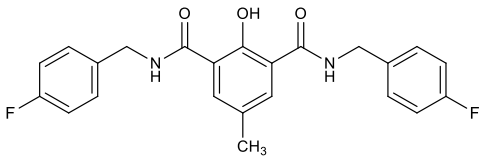

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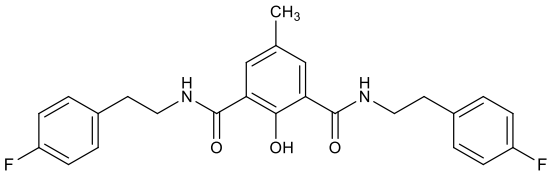

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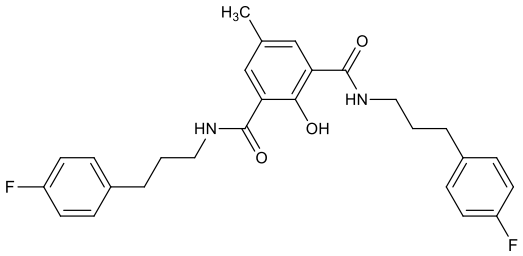
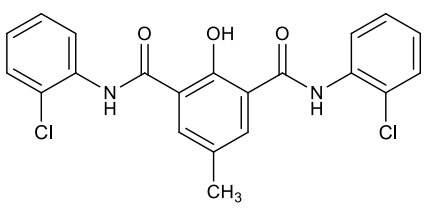
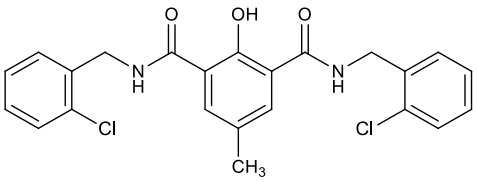


Compound 12



Compound 13



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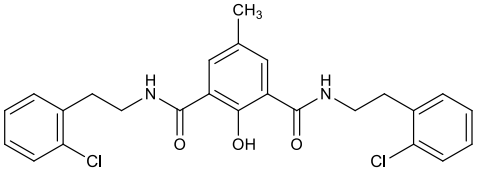

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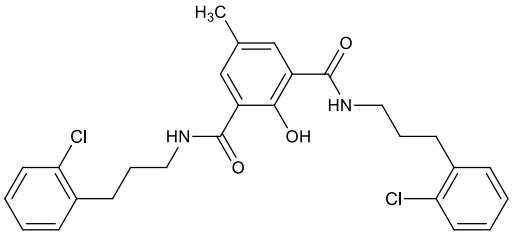

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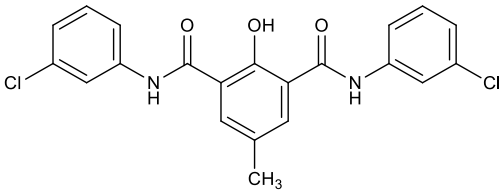

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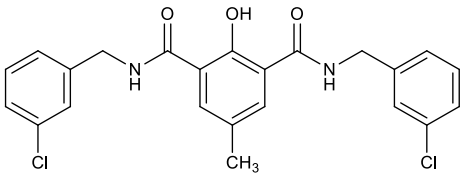

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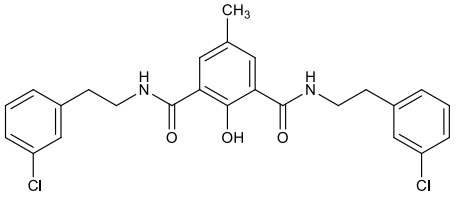

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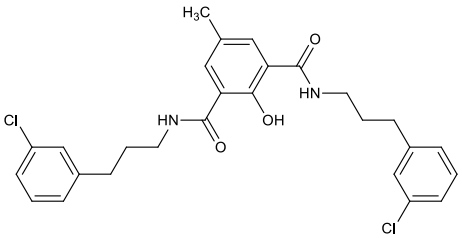

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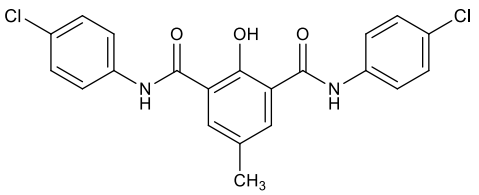

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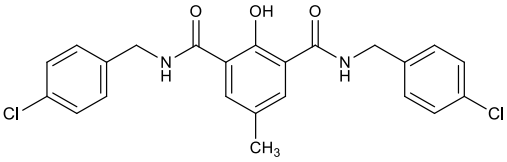

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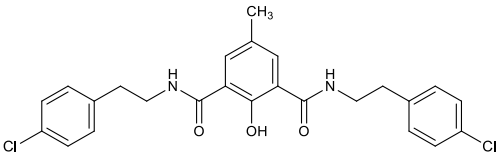

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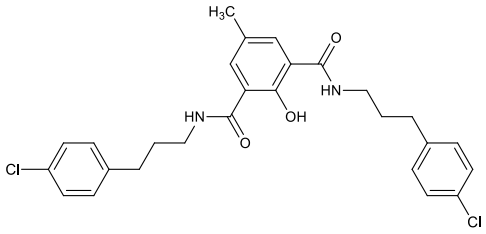

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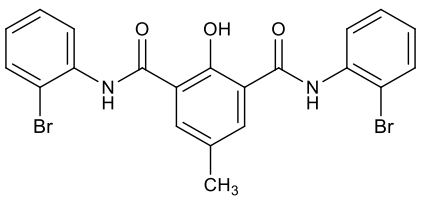

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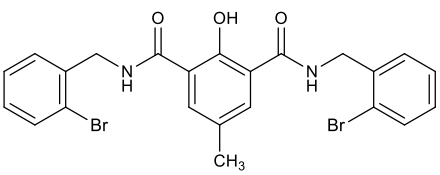

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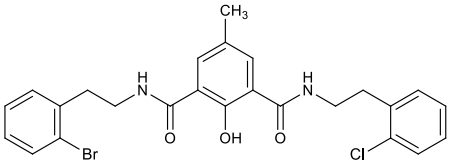

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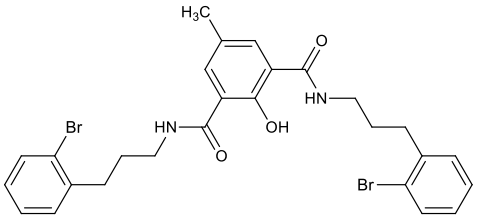

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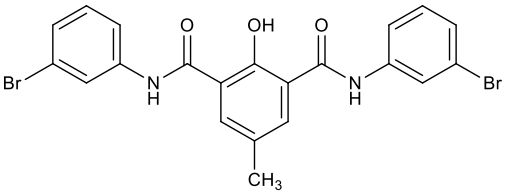

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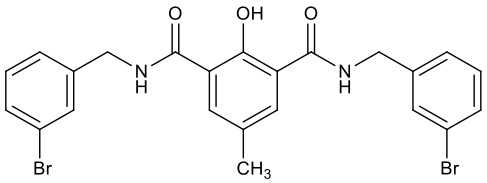

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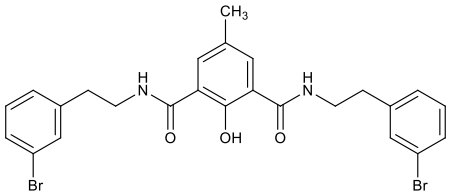

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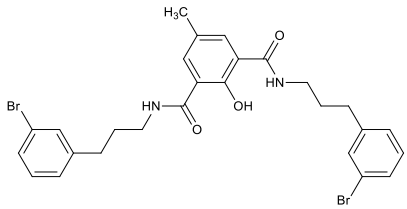

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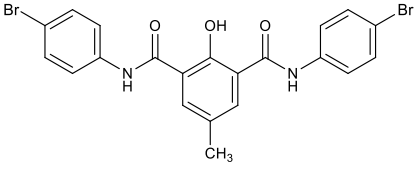

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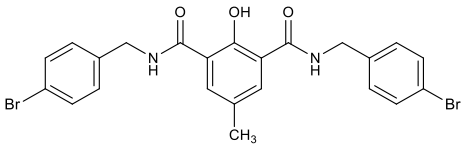

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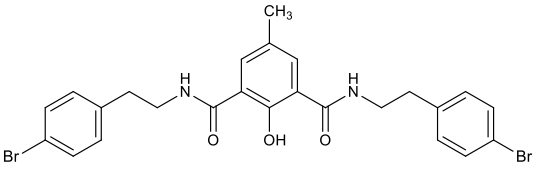

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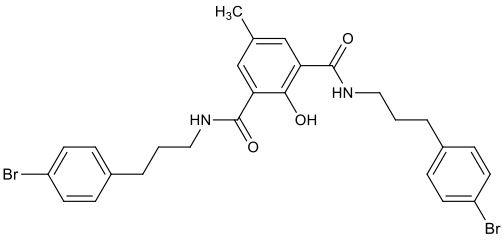

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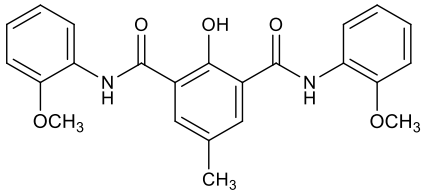

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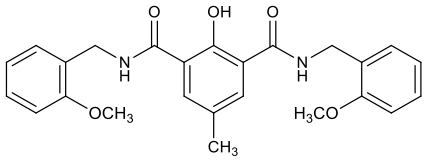

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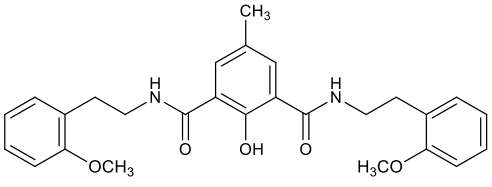

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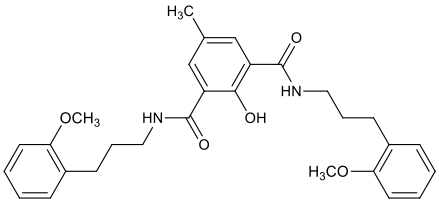

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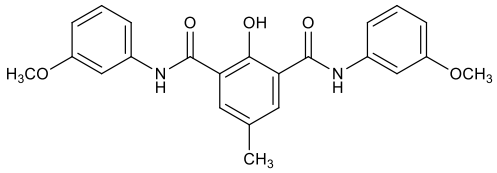

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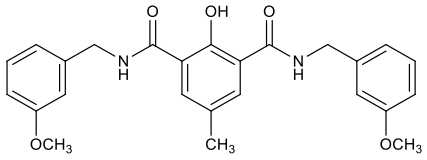

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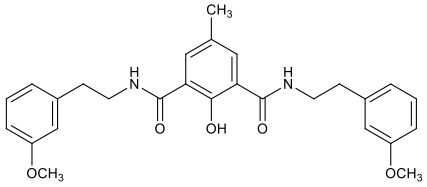

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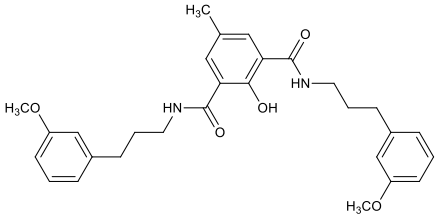

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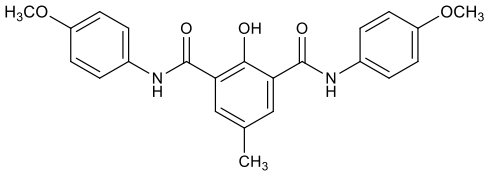

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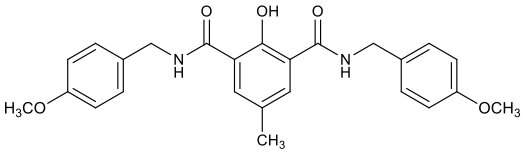

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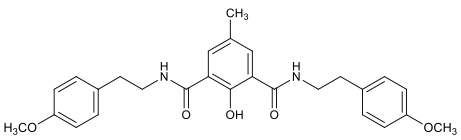

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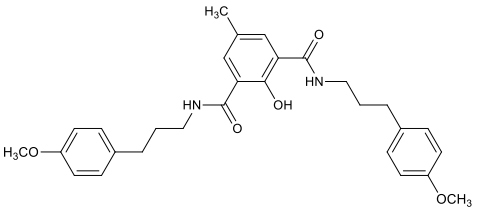

Compound 48

e-ISSN: 2687-6698 **Research Article**

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Non-Aqueous medium titrations of some 3-alkyl(aryl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones

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Abstract

A series of 3-alkyl(aryl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**1-9**) were titrated potentiometrically with tetrabutylammonium hydroxide in four non-aqueous solvents such as isopropyl alcohol, *tert*-butyl alcohol, acetonitrile, and *N*,*N*-dimethylformamide (DMF), and graphs were drawn for all cases. The half-neutralization potential values and the corresponding pK_a values were determined by the half neutralization method. Thus, the effects of solvents and molecular structure upon acidity were discussed.

Keywords: 1,2,4-triazol-5-one, Schiff base, acidity, potentiometric titrations, pK_a

1. Introduction

Several reports, involving the synthesis of some N-arylidenamino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives (Schiff bases), have been published up to date [1-9]. 1,2,4-Triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are investigated to show a broad spectrum of biological activities such as antimicrobial, antifungal, antihypertensive, hypoglycemic, analgesic, antiparasitic, antiviral, anti-inflammatory, hypocholesteremic, antitumor, antioxidant, and anti-HIV properties [3,5-16].

Weak acidic properties of 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one rings have been well known. In this study, some 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatived compounds were titrated potentiometrically with tetrabutylammonium hydroxide in non-aqueous solvents, and their pK_a values were determined [1-7,9,12,17-22]. We have previously reported the synthesis and potentiometric titrations of some new 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives in different non-aqueous mediums and determined the pK_a values of the compounds for each non-aqueous solvent [1-7,9,12,17-22]. Determination of p*K*a values of the active constituent of pharmaceutical preparations is valuable. Because distribution, transport behavior, bonding to receptors, and contributions to the metabolic behavior of the active constituents in the preparations depend on the ionization constant of the corresponding molecule [23-25].

The protonation constant of weak acidic compounds can be calculated by different methods. The potentiometric, chromatographic, and electrophoretic methods have been employed widely for this aim [26]. In the present work, the p*K*a values of some 1,2,4-triazole derivatives in non-aqueous media have been determined by using potentiometric titrations. These 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have been synthesized according to the reference in the literature [8].

2. Experimental

2.1. Preparation of compounds

The compounds **1-9** were prepared from the reactions of the corresponding 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones with 3-(2-metylbenzoxy)-4-methoxybenzaldehyde as described in the literature [8].

In this study, nine different 4,5-dihydro-1*H*-1,2,4-triazole derivatives [3-methyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**1**), 3-ethyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-

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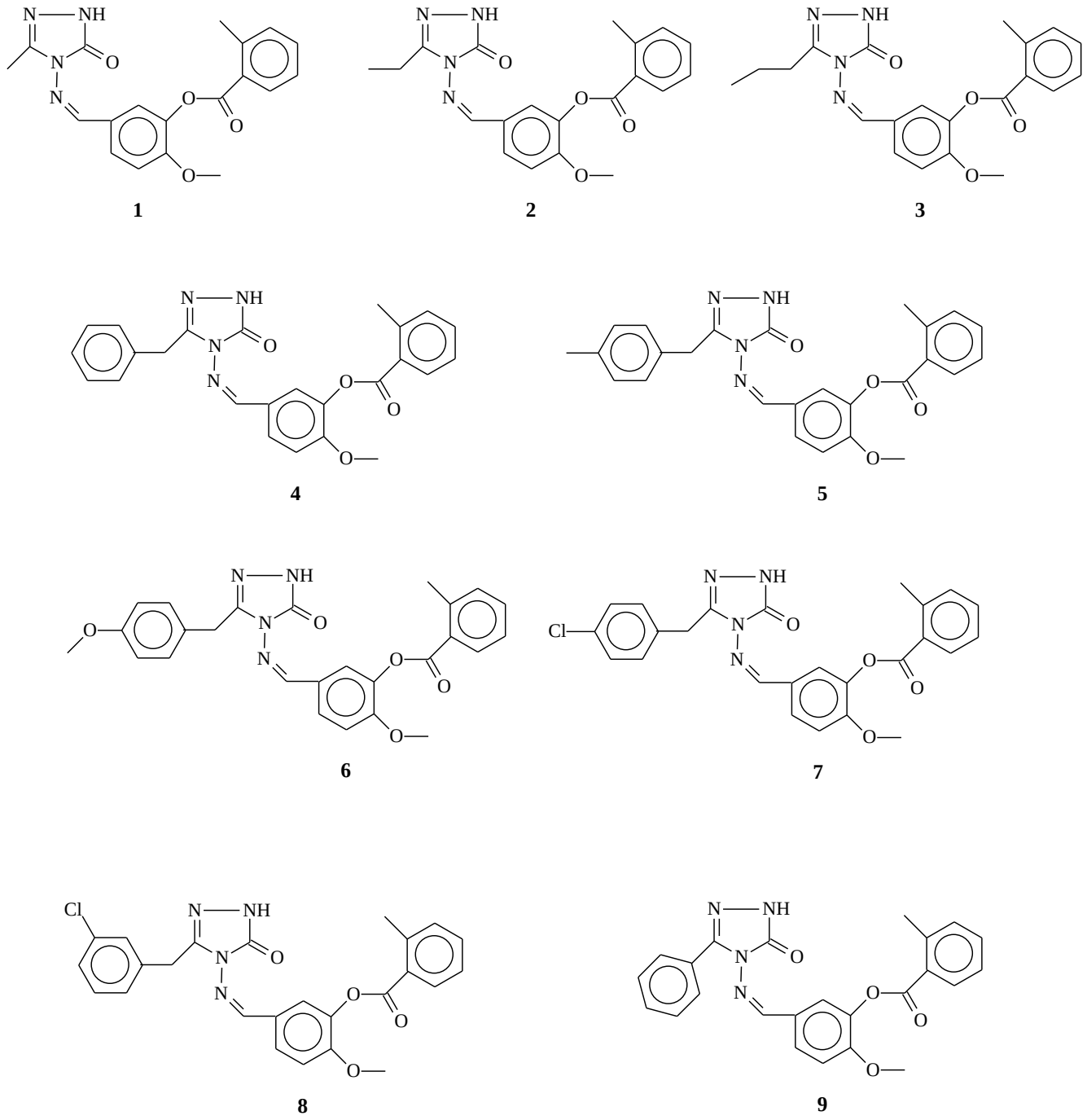

Figure 1. Studied 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives
1: 3-Methyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
2: 3-Ethyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
3: 3-(*n*-Propyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
4: 3-Benzyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
5: 3-(*p*-Methylbenzyl)-4-[3-(2-metylbenzoxy)-4-methoxy-benzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
6: 3-(*p*-Methoxybenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
7: 3-(*p*-Chlorobenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
8: 3-(*m*-Chlorobenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylidene-amino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one
9: 3-Phenyl-4-[3-(2-metylbenzoxy)-4-methoxy-benzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one

triazol-5-one (**2**), 3-(*n*-propyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**3**), 3-benzyl-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**4**), 3-(*p*-methylbenzyl)-4-[3-(2-metylbenzoxy)-4-methoxy-benzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**5**), 3-(*p*-methoxybenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**6**), 3-(*p*-chlorobenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**7**), 3-(*m*-chlorobenzyl)-4-[3-(2-metylbenzoxy)-4-methoxybenzylidene-amino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**8**), 3-phenyl-4-[3-(2-

metylbenzoxy)-4-methoxy-benzylideneamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**9**)] (Fig. 1) were titrated with tetrabutylammonium hydroxide (TBAH) in four non-aqueous solvents (*tert*-butyl alcohol, isopropyl alcohol, acetone, and *N*,*N*-dimethylformamide).

2.2. Potentiometric titrations

A Jenway 3040-model ion analyzer was used in potentiometric titration experiments. An Ingold pH electrode was employed in these experiments. The 0.001 M solution was separately prepared in each non-aqueous solvent for titration of each compound. The 0.05 M solution of TBAH in isopropyl alcohol because of widely its usage was employed as titrant. The mV values were recorded in the pH meter. Finally, the HNP values were found by drawing the mL (TBAH)-mV graphic.

3. Results and discussion

In this study, compounds **1-9** were titrated potentiometrically with TBAH in four non-aqueous solvents such as isopropyl (ε=19.4), *tert*-butyl alcohol (ε=12), acetone (ε=20.6) and *N*,*N*-dimethylformamide (ε=37) [3,6]. The mV values read in each titration were plotted against 0.05 M TBAH volumes (mL) added, and potentiometric titration curves were obtained for all the cases. From the titration curves, the HNP values were measured, and the corresponding p*K*a values were calculated.

The pH of weak acids can be found by using the Equation 1.

$$pH = pKa + log[A^-] / [HA] \qquad (1)$$

In Equation 1, pH equals p*K*a when [A$^-$] equals [HA], which is the half-neutralization point. Therefore, the *p*H values at the half-neutralization points were determined as p*K*a.

According to the dielectric permittivity of the solvents, the acidity ranking might be expected to be as follows: *N*,*N*-dimethylformamide (ε=37) > acetone (ε=20.6) > isopropyl alcohol (ε=19.4) > *tert*-butyl alcohol (ε=12) [3,6]. However, as seen in Table 1, the acidic arrangement for compounds **3** and **6** is: isopropyl alcohol > acetone > *tert*-butyl alcohol > *N*,*N*-dimethylformamide, for compounds **4** and **9**, it is: isopropyl alcohol > *tert*-butyl alcohol > acetone > *N*,*N*-dimethylformamide, for compounds **5** and **8**, it is: *N*,*N*-dimethylformamide > acetone, for compound **1**, it is: *tert*-butyl alcohol > isopropyl alcohol > *N*,*N*-dimethylformamide > acetone, and for compound, **2** it is: acetone > isopropyl alcohol > *N*,*N*-dimethylformamide, while the order for compound **7** is: *tert*-butyl alcohol > acetone > *N*,*N*-dimethylformamide.

As seen in Table 1, in isopropyl alcohol, compounds **3, 4**, and **6**, in *tert*-butyl alcohol **1**, **7** and **9**, in DMF, compounds **5** and **8**, in acetone, compound **2** show the strongest acidic properties, while compounds **2-4**, **6**, **7** and **9** show the weakest acidic properties in *N*,*N*-dimethylformamide (acetone for compounds **1**, **5** and **8**). This situation can be result from the hydrogen bonding between the negative ions formed and the solvent molecules in the amphiprotic neutral solvents [1-7,9,12,17-22].

The half-neutralization potential (HNP) values and the corresponding p*K*a values of compounds **1-9**, obtained from the potentiometric titrations with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, acetone, and DMF, are presented in Table 1.

Table 1. The HNP and the corresponding p*K*a values of compounds **1-9** in isopropyl alcohol, tert-butyl alcohol, DMF, and acetone at 25 °C

Compound No	DMF		Acetone		tert-Butyl alcohol		Isopropyl alcohol	
	HNP (mV)	p*K*a	HNP (mV)	p*K*a	HNP (mV)	p*K*a	HNP (mV)	p*K*a
1	-334	14.32	-478	17.12	-185	10.71	-230	13.16
2	-315	14.36	-226	11.69	-	-	-277	13.48
3	-289	13.37	-215	11.55	-246	11.83	-152	10.34
4	-180	12.53	-159	11.21	-222	11.00	-106	9.16
5	-326	14.62	-418	16.53	-	-	-	-
6	-428	16.96	-195	11.56	-267	12.97	-148	10.08
7	-370	14.18	-218	12.18	-85	7.24	-	-
8	-279	13.20	-324	13.54	-	-	-	-
9	-336	14.99	-243	11.57	-209	10.12	-248	11.14

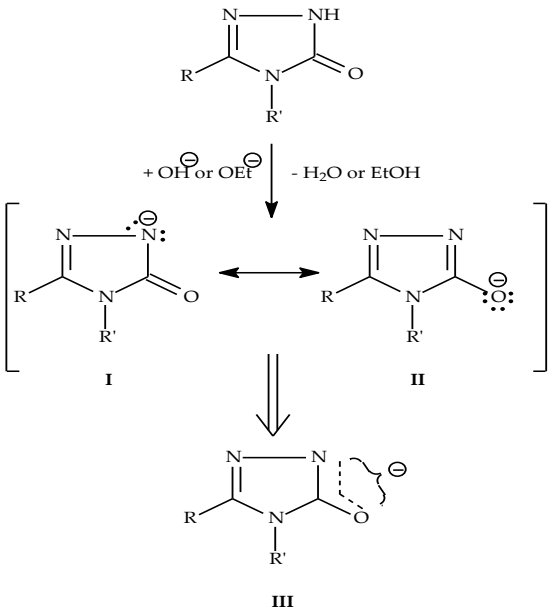

Figure 2. Resonance structures

As seen in Table 1, for compound **2** in *tert*-butyl alcohol, compound **5** in *tert*-butyl alcohol and isopropyl alcohol, compound **7** in isopropyl alcohol, compound **8** in acetone, *tert*-butyl alcohol, and isopropyl alcohol, compound **3e** in *tert*-butyl alcohol and isopropyl alcohol, the HNP values and the corresponding p*K*a values have not been obtained.

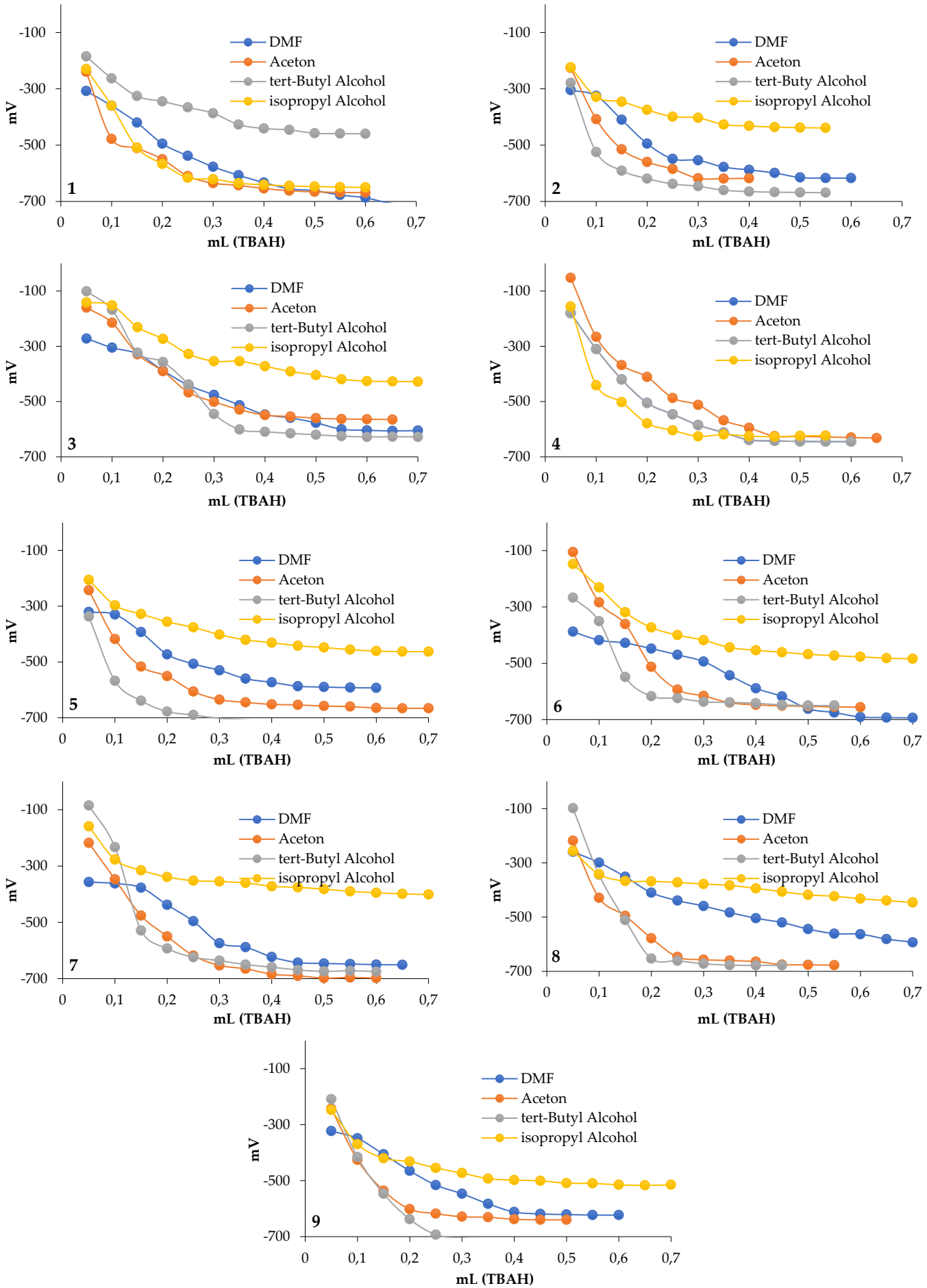

Figure 3. Potentiometric titration curves of 0.001 M solutions of compounds **1-9** titrated with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, acetone, and *N*,*N*-dimethylformamide at 25°C

Acidic properties of a compound depend on some structural and environmental factors. The two most important factors from these are the solvent effect and molecular structure of the compound [1-7,9,12,17-22,27]. Table 1 shows that the HNP values and corresponding p*K*a values determined from the potentiometric titration experiments depend on the non-aqueous solvents used and the substituents at C-3, in the 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring. This situation may be attributed to resonance structures as seen in Fig. 2.

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Synthesis and analytical applications of thiosemicarbazide derivative

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Abstract

1-(4-*tert*-Butylphenyl)-4-(4-trifluoromethylphenyl)carbonylthiosemicarbazide (OBS) was synthesized by reaction of 4-*tert*-butylbenzoic hydrazide with 4-(trifluoromethyl)phenylisothiocyanate. The influence of many metal cations on the spectroscopic properties of the synthesized compound was investigated in DMSO-H_2O (1:1) by means of emission spectrometry. The influence of a series of metal ions including Cu^{2+}, Co^{2+}, Al^{3+}, Cr^{3+}, Fe^{3+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Ag^{+}, Ba^{2+}, Mo^{3+}, Se^{2+}, Ca^{2+}, Sn^{4+}, Sb^{3+}, Na^{+}, K^{+}, Li^{+}, Sc^{3+}, Sr^{2+}, As^{3+}, Be^{2+} and Mg^{2+} on the spectroscopic properties of the ligand was investigated by means of emission spectrometry. The compound was selectively complexed with Fe^{3+} among many metal ions. The complex stoichiometry and the stability constant were determined by fluorometric measurements. The ligand including thiosemicarbazide functional group (TSC) showed sensitivity for Fe^{3+} ion with a linear range between 0.5 and 5.5 mg/L. The new method was employed in the determination of iron in the sandy-soil reference material. A modified standard addition method was applied to remove the matrix effect. Detection and quantification limits were 0.07 and 0.214 mg/L, respectively. The simple and cost-effective method can be applied to soil samples.

Keywords: Thiosemicarbazides, Fe^{3+}, fluorescence measurements, complex stoichiometry

1. Introduction

Metals are important components of natural life [1]. Most of the metals are also essential for the human body. For example, the effects of iron on metabolism are still under investigation [2]. Iron has very important effects on human health. Because iron participates in many metabolic processes such as oxygen transport, deoxyribonucleic acid (DNA) synthesis, electron transport, hemoglobin production. But since iron can form free radicals, its amount in body tissues must be controlled and regulated. Otherwise, it causes many diseases, such as excessive iron loading from anemia. Because of this, the amount of iron that should be found in the body is also limited. Their deficiencies or excesses cause serious health problems. Therefore, there is always a need to develop a method for the detection and detection of metals in biological or environmental samples [3].

AAS and ICP methods for sensitive metal determination are generally expensive and time-consuming methods. Also, they require sample clean-up, preconcentration, and separation of interfering species before the analysis [4-9]. Namely, these methods are not fast and simple. Therefore, the development of alternative simple and fast methods is important to determine metal ions.

For metal determinations, fluoroionophore compounds have been used as a selective and sensitive analytical reagent. The ionophore part forms selectively a complex with target metal ion and it provides the selectivity of the method. The fluorophore part signals the complexation between metal ion and ionophore part through its fluorescence properties [10]. The high fluorescence property of the fluorophore group is desired for a highly sensitive analytical method. Therefore, the fluoroionophore comprising an appropriate ionophore and an effective fluorophore can be used to determine the target ion selectively and sensitively. From this point of view, many fluoroionophore compounds synthesized for metal determination are available in the literature [11-14].

Thiosemicarbazides (TSC) having the -CO-NH-NH-CS-NH- functional group are generally synthesized from reactions of hydrazides with isothiocyanates in different organic solvents [15]. TSC are used both as pharmacological active compound sand as starting compounds for the synthesis of diverse bioactive

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heterocyclic compounds such as 1,2,4-triazole, 1,3,4-oxadiazole, 1,3,4-thiadiazole and thiazolinone [16-19]. They are also showed a wide range of various biological activities like antimicrobial [20], antiviral [21], anticancer [22], anti-inflammatory [23], anti-tubercular [24], topoisomerase IV and urease inhibitors [25,26]. Moreover, thiosemicarbazides can easily form metal complexes with different transition metals due to their donor groups such as nitrogen, oxygen, and sulfur [27]. Also, thiosemicarbazide has strong coordinating abilities with metal ions and is often used as the mother molecule for the design of metal-ion sensors. For this reason, there are many studies in the literature [28-29]. Recently, metal determination has been made by synthesizing nano-points from thiosemicarbazides with their exceptional optical stability and ease of functionality [30-31].

In this study, we report 2 ligands with thiosemicarbazide and semicarbazide functional groups. Fluorescence quenching of ligands against increasing metal ions was determined by spectrofluorimetric titrations in DMSO-H_2O (1/1). A simple spectrofluorimetric method was developed to determine Fe(III) from sand-soil reference material using a thiosemicarbazide compound derived from 4-tert-Butylbenzoic hydrazide and 4-(trifluoromethyl)phenyl isothiocyanate as the analytical ligand.

2. Experimental

2.1. Chemicals

Chemical reagents purchased from companies such as Sigma-Aldrich, Merck, Alfa Aesar and Acros were used without further purification. The standard solutions of cations (1000 mg/L, Merck) were used to prepare working solutions. A sandy-soil standard reference material (CRM-SA-C,) was purchased from High-Purity Standards, Inc. Dimethyl sulfoxide (DMSO) from Merck (spectrometric grade) was used as a solvent for fluorescence measurements.

2.2. Apparatus

Melting points were recorded using Thermo Scientific digital 9200 melting point apparatus. Proton-NMR and Carbon-13 NMR (APT) spectra were recorded on a Bruker Avance II 400 MHz NMR instrument using Dimethyl sulfoxide-d_6. A PTI Spectrofluorometer (QM-4/2006) was used for all fluorescence measurements.

2.3. Synthesis of 1-(4-*tert*-butylphenyl)-4-(4-trifluoromethylphenyl) carbonyl thiosemicarbazide (OBS)

4-*tert*-Butylbenzoic hydrazide (10 mmol) and 4-(trifluoromethyl)phenyl isothiocyanate (10 mmol) were refluxed in absolute ethanol for 40 min and, at the end of this period a white precipitate formed. The precipitated solid was filtered, washed with hot petroleum ether (35-60 °C), dried, and recrystallized from ethanol. Yield: %94; mp. 228-229 °C. FTIR-ATR (ν, cm^{-1}): 3301, 3220 (NH), 1641 (C=O), 1242 (C=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ:3.83 (s, 3H, OCH_3), Ar-H: [7.06 (d, 2H, J = 8.0 Hz), 7.69-7.76 (m, 4H), 7.95(d, 2H, J = 8.0 Hz)], 9.95 and 10.01 (s, 2H, 2NH), 10.48 (NH); ^{13}C (APT) NMR (100 MHz, DMSO-d_6) δ: 55.90 (OCH_2), Ar-C: [113.99 (CH), 125.01, 125.44 (2CH), 126.21 (2CH), 130.35 (2CH), 143,58, 162.61], 120.77 (CF_3), 165.88 (C=O), 181.46 (C=S).

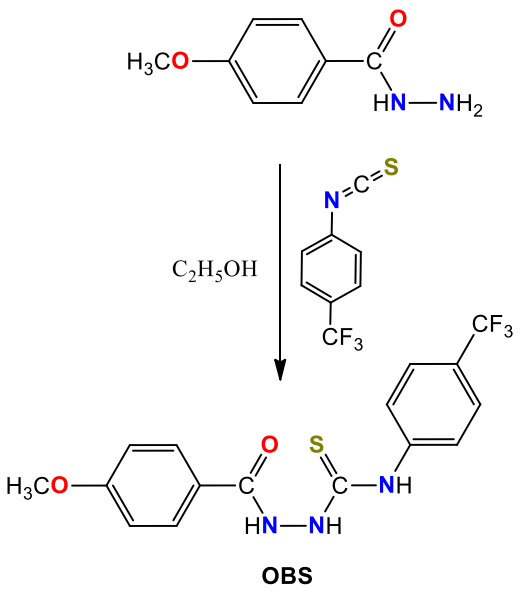
2.4. Analytical measurements

Fluorescence spectra of the ligands containing 10 M equivalents of Cu^{2+}, Co^{2+}, Al^{3+}, Cr^{3+}, Fe^{3+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Ag^{+}, Ba^{2+}, Mo^{3+}, Se^{2+}, Ca^{2+}, Sn^{4+}, Sb^{3+}, Na^{+}, K^{+}, Li^{+}, Sc^{3+}, Sr^{2+}, As^{3+}, Be^{2+} and Mg^{2+} were recorded using 1 cm quartz cell. Different organic solvents were tested to determine the best solvent as ligand solvent. Diluted solutions of the ligands in DMSO were used in spectro fluorimetric titrations. 1-(4-*tert*-butylphenyl)4-(4-trifluoromethylphenyl)carbonylthiosemicarbazide (OBS) was 1.0 × 10^{-5} M in spectrofluorimetric measurements, and the excitation wavelength was 355 nm for this ligand. Fluorescence emission spectra were recorded in the range of 300-600 nm with the slit width of 1.0 nm. The molar-ratio method was employed to determine the stoichiometry of the complex. The stability constant of complex was calculated according to the known procedure [32]. A kind of standard addition method [33-37] was used to determine Fe^{3+} in the spiked sand-soil reference material with OBS

3. Results and discussion

3.1. Synthesis and characterization

1-(4-*tert*-butylphenyl)4-(4-trifluoromethylphenyl)-carbonylthiosemicarbazide was synthesized by reaction of 4-*tert*-butylbenzoic hydrazide with 4-(trifluoromethyl)phenyl isothiocyanate (Scheme 1). The IR spectra of the compound have C=O, C=S and N-H stretching bands of thiosemicarbazide group at 1641 cm^{-1}, 1242 cm^{-1} and 330, 3220 cm^{-1}, respectively. In the ^{1}H-NMR spectra, resonances assigned to the three N-H protons of the compound were detected at 9.95 and 10.01 ppm (s, 2H, NHCSMH) and 10.48 ppm (s, 1H, NH-C=O) respectively. In the ^{13}C-NMR spectrum, signals of C=O and C=S carbons were observed at 165.88 and 181.46 ppm.



Scheme 1. The synthesis of OBS

3.2. The effect of ions on the fluorescence spectra

The effect of 10 equivalent excess of Cu^{2+}, Co^{2+}, Al^{3+}, Cr^{3+},Fe^{3+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Ag^{+} , Ba^{2+} , Mo^{3+}, Se, Ca^{2+},Sn^{4+},Sb^{3+}, Na^{+}, K^{+}, Li^{+}, Sc^{3+}, Sr^{2+}, As^{3+}, Be^{2+} and Mg^{2+} ions on the fluorescence spectra of the compounds in DMSO : H_2O (1:1) was investigated. As seen from Figs. 1-4, Fe^{3+} ion cause pronounced quenching in the fluorescence spectra of QBS ligand.

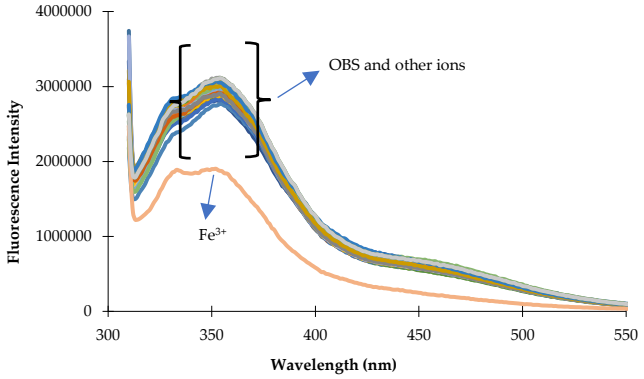


Figure 1. The effect of cations on the fluorescence spectra of OBS. OBS concentration = 1 × 10^{-5} M. Ion concentration = 1 × 10^{-4} M

Fig. 1 shows the effect of cations on fluorescence spectra of OBS compound with thiosemicarbazide functional group. OBS shows selectivity for Fe^{3+} (Fig.2). The other cations did not cause any changes in the fluorescence intensity of OBS at 355 nm (Fig.2).

For Fe^{3+} fluorimetric titrations have been performed with OBS to disclose metal-ligand interaction. Fig. 3 shows the regular fluorescence quenching with increasing Fe^{3+} concentration in the case of OBS. The change in the absorbance at 355 nm has been given in Fig. 4 inset. As seen from Fig. 4 inset, the linearity has continued until the Fe^{3+} concentration of 5.5 mg/L. A deviation from Beer's law has been observed above this concentration. The experiments have shown that this linear response can be used to determine Fe^{3+} concentration in sandy soil samples that have suitable Fe^{3+} content.

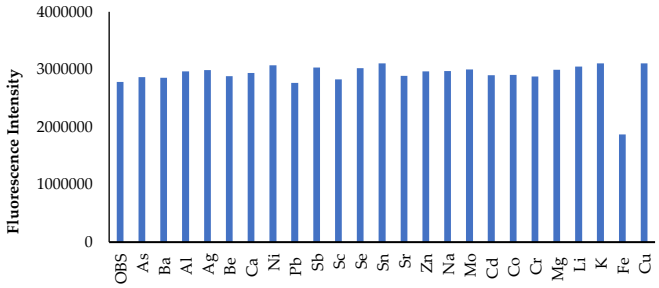


Figure 2. The effect of ions on the fluorescence spectra of OBS at 355 nm. OBS concentration = 1 × 10^{-5} M. Ion concentration = 1 × 10^{-4} M

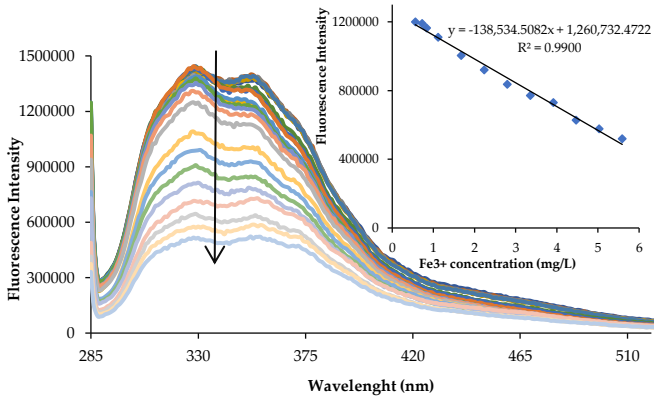


Figure 3. The quenching in the fluorescence spectra of OBS with the increasing Fe^{3+}concentration. Inset: Linear concentration range to determine Fe^{3+}. Measurements were carried out at 355 nm. OBS concentration = 1×10^{-5} M

3.3. The proposed method for Fe^{3+} determination

Acceptable recovery results were obtained with the modified standard addition method. The experimental details for the used modified standard addition method were given in the previous studies [36,37]. A constant amount of Fe^{3+} (1.0 mg/L), 2 mL of ligand (1 × 10^{-5} M), and an aliquot spiked sample (1.5 mg/L) were added to all tubes. The sample was not added to the first tube while increasing amounts of Fe^{3+} were added to the third and next tubes. The final volumes were made 2 mL with deionized water. The fluorescence intensity of all solutions was measured at 355 nm by exciting at 300 nm. The Fe^{3+} concentration was calculated from Equation 1

$$C_x=(F-F_0)/m \qquad (1)$$

where C_x is the Fe^{3+} concentration of the sample, F and F_0 are the fluorescence intensities of the first and second tubes, respectively, and m is the slope of the modified standard addition graph. The difference between F and F_0 is related to the Fe^{3+} concentration of the sample in the tubes. Fig. 4 shows the modified standard addition

graph for the determination of Fe^{3+} (1.5 mg/L) in spiked sample.

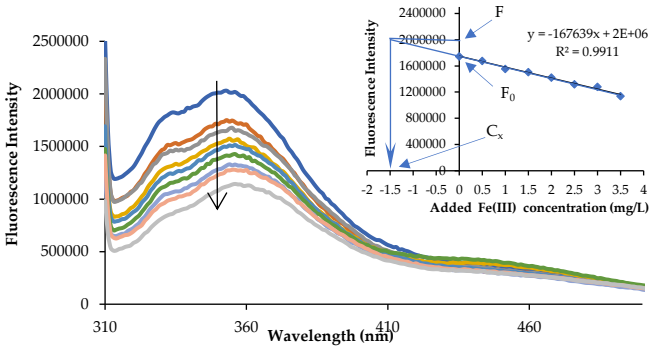


Figure 4. Changes in the fluorescence spectra of OBS over added Fe^{3+} concentration in the modified standard addition experiments. Ligand concentration = 1×10^{-5} M. Excitation at 300 nm. Measurements were carried out at 355 nm

Excitation wavelengths between 300 and 400 nm were used to obtain maximum emission intensity with OBS. The maximum fluorescence intensity was obtained when the ligand is excited with 300 nm. Excitation at 300 nm of OBS (1×10^{-5} M) gave two suitable emission bands at 333 and 355 nm in DMSO:water (1:1). Therefore, 300 nm was selected as the excitation wavelength.

The measurements were carried out in two characteristic emission wavelengths (333 and 355 nm) to determine Fe^{3+} in the spiked samples with acceptable recovery results. The recovery results were acceptable for the measurements at 355 nm. Therefore, this wavelength was selected as the measurement emission wavelength.

The optimization of constant analyte concentration is the determining stage to work the modified standard addition method according to our recent experiences [36, 37]. Also, in this study, various Fe(III) concentrations (1.0-2.0 mg/L) were tested to obtain accurate results with the modified standard addition method. The optimum constant Fe(III) concentration was found as 1.5 mg/L.

Modified standard addition experiments were carried out using the ligand solutions in the range of 5.0×10^{-5} to 1.0×10^{-6} M with increasing concentrations of Fe(III). The values of fluorescence intensity at 355 nm were plotted against Fe(III) concentration. The R^2 value of 0.9911 was obtained for a ligand concentration of 1.0×10^{-5} M (Fig.4). Therefore, in further works, this concentration was used.

A linear response of the fluorescence intensity as a function of Fe^{3+} concentration at 355 nm was observed between 0.5 mg/L and 5.5 mg/L. LOD (3xSd/m) and LOQ values (9xSd/m) were determined using the standard deviation of eleven measurements of the blank response (Sd) and the slope of the calibration line (m) according to the IUPAC recommendations.

To investigate the accuracy of the proposed method, known amount of Fe(III) were added to samples and the recovery studies were carried out. The modified standard addition method was used in the spiked samples. The % error was below 5% for concentration level (1.5 mg/L). The recovery % value was 96.01 for 1.5 mg/L Fe(III) concentration.

The intra-day and inter-day precision was estimated by analysis three independent samples spiking with 1.5 mg/L Fe(III). The precision was expressed as relative standard deviation (RSD %) and it was 7.90 for the intra-day measurements. Inter-day precision was 3.70%.

Table 1. Analytical performance data of the proposed method for Fe^{3+} determination

Excitation wavelength (nm)	300
Emission wavelength (nm)	355
Limit of detection (LOD) (mg/L)	0.07
Limit of quantification (LOQ) (mg/L)	0.214
Linear range (mg/L-mg/L)	0.5- 5.5
Constant Fe^{3+} concentration (mg/L)	1.5
Ligand concentration (mol/L)	1.0×10^{-5}
Ligand volume (mL)	2.0
Total volume (mL)	4.0
Solvent	DMSO:water (1:1)
Time before measurement (minutes)	1-2
Correlation coefficient (R^2)	0.9911

3.4. Advantages of the proposed method

Table 1 gives analytical performance data of the proposed method for Fe^{3+} determination. The proposed method can be directly applied to water samples with the Fe^{3+} concentration above 1.5 mg/L. There is no need either for the separation of Fe^{3+} ion from sandy soil sample or sample treatment such as pre concentration stage before the measurement. To remove possible matrix effects, a known modified standard addition method is employed. A time of 1-2 min was enough before measurement to obtain a stable fluorescence response. These properties make the proposed method simple and timesaving. Moreover, the method is partially environmentally friendly because the ligand is diluted with water. Spectrofluorometric methods are much cheaper compared with atomic methods. Consequently, the proposed method is timesaving, eco-friendly, simple and cheap when compared to alternative methods in the literature.

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Supplementary materials

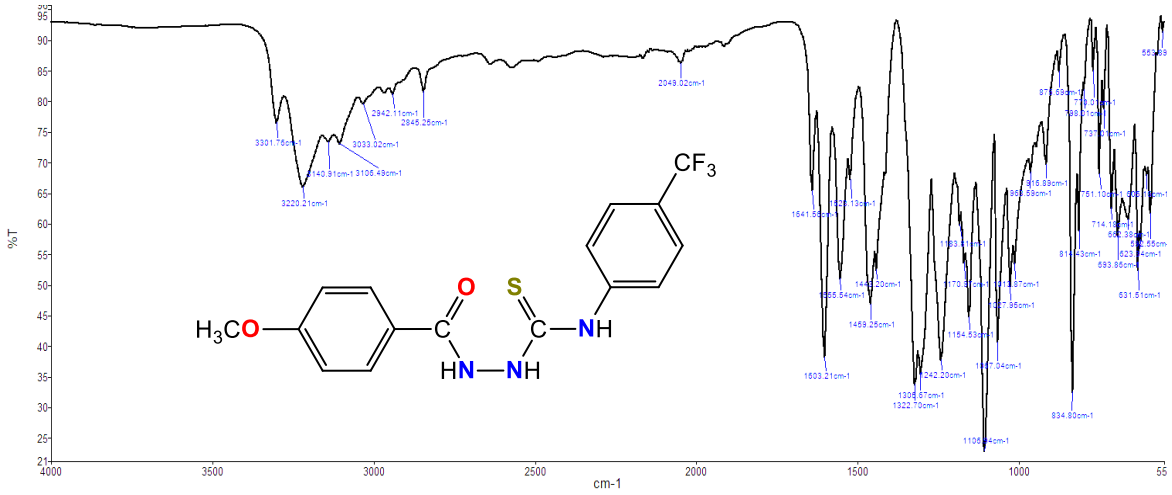

Figure 1. IR spectrum of OBS.

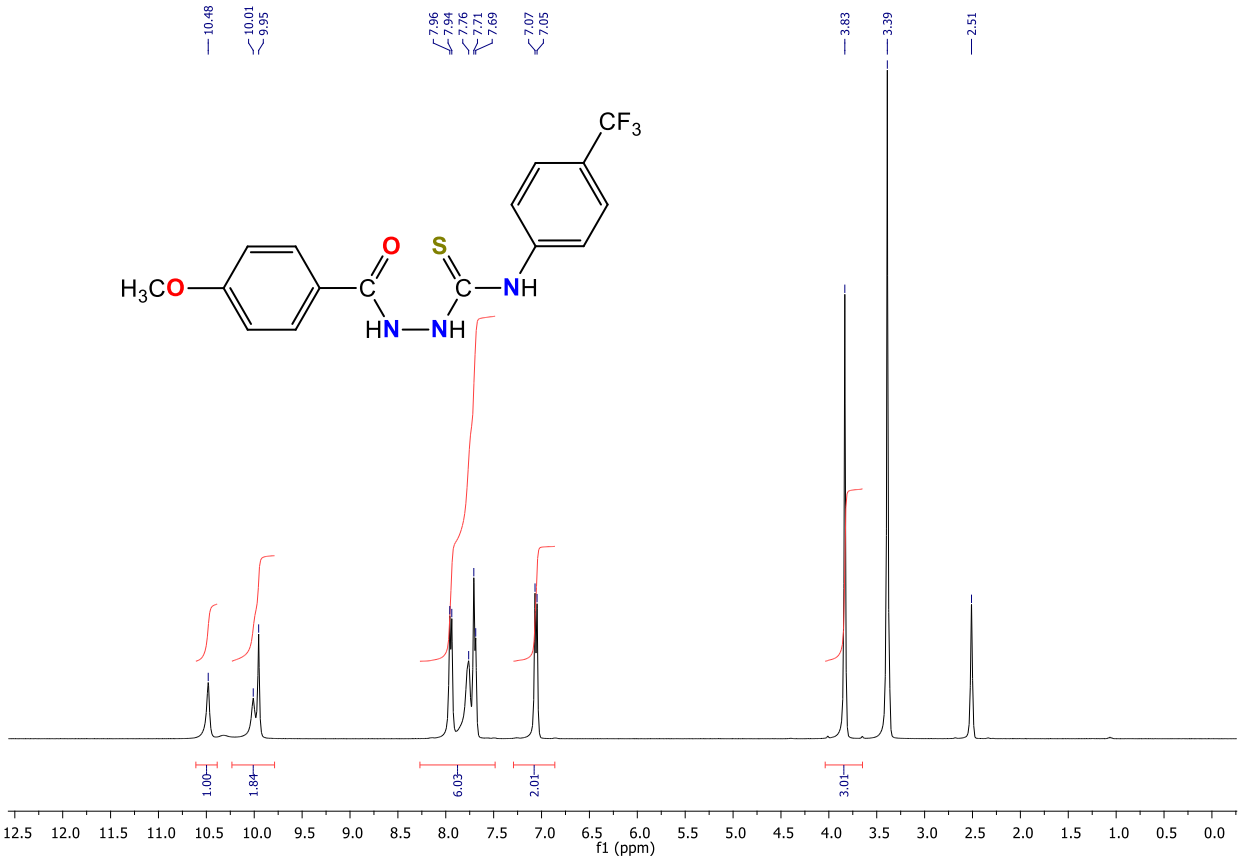

Figure 2. ^{1}H NMR spectrum of OBS.

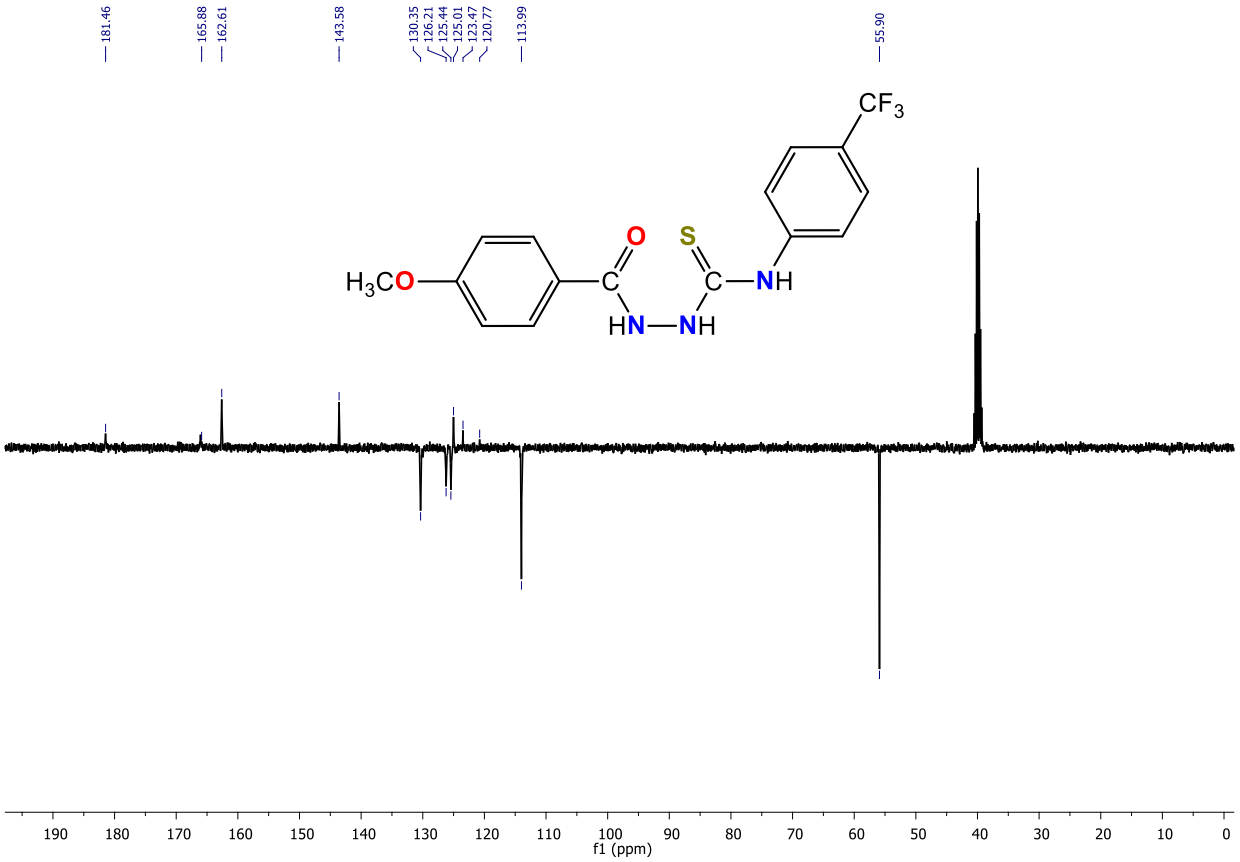

Figure 3. ^{13}C NMR spectrum of OBS.

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Comparison of different extraction methods for total phenolic content and antioxidant activity of dried *Diospyros lotus* L fruits

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Abstract

The yield, total phenolic content, and antioxidant activity values of the extracts obtained from the dried fruits of the Diospyros lotus L. plant by using Soxhlet (SXE), ultrasound-assisted extraction (UBE), and hot solvent extraction (HME) techniques with methanol were investigated. The highest extraction yield was obtained from HME experiments with 50.67 ± 0.63% and UBE with 49.50 ± 1.05%, respectively. While the extract obtained by the UBE technique showed a lower TPC value (1464 ± 57 mg GAE/100 g original sample) compared to the extracts obtained from the other two techniques, it showed higher antioxidant activity values than that of the HME technique. While these values were determined as 192.53 ± 4.45 and 273.10 ± 34.79 mg/mL (SC_{50}, lower is better) for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay, 0.65 ± 0.04 and 0.25 ± 0.04 g TEAC/100 g air-dried sample for cupric ion reducing antioxidant activity (CUPRAC) assay, respectively. Both the UBE and HME techniques have clearly demonstrated that they are more advantageous than conventional Soxhlet extraction for simplicity of application, reduced solvent consumption, extraction of thermally sensitive compounds, and shortened extraction times.

Keywords: *Diospyros lotus* L., total phenolic content, antioxidant activity, Soxhlet extraction, ultrasound-assisted extraction, hot methanol extraction

1. Introduction

In recent years, there has been an increasing number of studies on the extraction of antioxidant compounds from natural origins, isolation of active ingredients, or the use of these extracts directly instead of synthetic antioxidants in order to extend the shelf life of foodstuffs. The driving force behind this situation is the research results that synthetic antioxidants may have some health-related drawbacks [1].

Compounds with antioxidant effects are usually found in low concentrations in their natural sources, so the number of antioxidants supplied with the amount of food taken in normal dietary meals is not able to reach the desired levels. Numerous studies have been brought to the literature by various research groups in order to eliminate this deficiency and to increase the concentration of antioxidant compounds by extracting them from natural products by various methods, to calculate the amount of the original product required for the intake of sufficient antioxidants, and to determine the optimum conditions for extraction. One of the pillars of the studies carried out in this direction is the development and use of new and more effective extraction techniques. Some of the new techniques used alongside the traditional Soxhlet technique (which is considered as a reference technique) in bioactive compound extraction from natural products are (1) Supercritical Extraction (SCE), (2) Ultrasound-Assisted Extraction (UAE), (3) Hot Solvent Extraction (HSE) and Microwave-Assisted Extraction (MAE) [2-6].

Locally, dried fruits of *D. lotus* L are consumed directly (especially in Artvin-Yusufeli) or used to sweeten tea in Eastern provinces (Erzurum, Kars). Jam is also made from ripe fruits in these regions. It is known to have a constipation effect [7]. The fruits of the plant have been investigated by different research groups in terms of antioxidant [8-10], anticancer properties [8], phenolic content [10], fatty acid composition [11]. However, none of these studies is a comparative study

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based on extraction techniques. Almost all of them were carried out by using traditional percolation and/or refluxing techniques, and no studies were found with new extraction techniques. Thus, the aim of the study is to compare the effects of three different extraction techniques (Soxhlet, ultrasound-assisted and hot solvent extractions) applied to dried and ground fruits of the *D. lotus* L. plant using methanol solvent on the total phenolic content and antioxidant activities of the extracts.

2. Materials and methods

2.1. Material and sample preparation

D. lotus (Black Date Persimmon) is generally grown in the North and North-East regions of Turkey for its edible fruit. The plant is a 10-15 m high tree with simple leaves, reddish or greenish-white flowers, and deciduous in winter. Its fruit is up to 15 mm in diameter, yellowish or bluish-black in color, and it is a spherical-shaped drupe (Fig. 1).

Figure 1. Air-dried fruits of *D. lotus* L (from Dr. F. Akdeniz archive)

Plant material was obtained as dried fruit from commercial sources of Trabzon city in Turkey for this study. Dried fruits were pitted, ground in an IKA A11 basic model laboratory mill, and stored in colored storage bottles at 4°C.

2.2. Extraction procedures

In this study, two techniques that have recently attracted attention in the extraction of antioxidant active substances, Ultrasound-Assisted Extraction (UAE) (an ultrasonic bath was used in the study, thus, it is referred to as UBE) and Hot Solvent Extraction (HSE) techniques were used and the results were compared to those of obtained in the traditional Soxhlet Extraction (SXE) technique. Methanol, which is known to be effective in dissolving phenolic antioxidant compounds, was used as the extraction solvent. Total phenolic contents (TPCs) of the extracts were determined according to Folin Ciocalteu's method [12]. Antioxidant activities were determined according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method [13] and the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) determination method which is a relatively new method developed by Apak et al. [14].

For Soxhlet extraction experiments, approximately 5 g of the air-dried matter was weighed with 0.1 mg precision and placed in the extraction cartridge, then it was extracted for 6 hours (approximately 36 siphons) using 150-200 mL of HPLC grade methanol (Sigma-Aldrich, Germany). In ultrasonic bath extractions (UBE), 5 g of substance was weighed with 0.1 mg precision and placed in a conical flask and 100 mL of solvent (methanol) was added each time. After they were mixed thoroughly, it was placed in a Bandelin Sonorex (Germany) model ultrasonic bath and extracted for 20, 10, and 5 min (in total 35 min), respectively, at a frequency of 35 kHz. In hot methanol extractions (HME), 0.5 g sample weighed with 0.1 mg sensitivity was placed in an autoclave with a volume of 75 ± 1 mL made of 316 stainless steel (autoclave described in detail elsewhere [15]. 15 mL of solvent was added to it and extracted at 100 ± 3°C for an hour. All experiments were carried out in triplicate.

The solvent of the extracts obtained was removed in a rotary evaporator, and the extract yields were calculated in percentage. Later, stock solutions were obtained by dissolving all extracts in methanol again and these solutions were kept at + 4°C in a refrigerator for total phenolic substance and antioxidant activity determination experiments.

2.3. Total phenolic content (TPC) and antioxidant activity assays

TPCs of the extracts were determined according to Slinkard and Singleton's method [12] with slight modifications applying the Folin-Ciocalteu reagent. For this purpose, 2.5 mL of distilled water was added to 50 µL of the solution with a concentration of 1 mg/mL prepared by diluting with the solvent used from the stock solutions. From 0.2 N solution prepared from original purchased Folin-Ciocalteau solution of 2 N by diluting at a volume ratio of 1:10, 250 µL was added to it, vortexed, and kept at room temperature for 3 minutes. An aliquot of 750 µL of a 7.5% (w/w) Na_2CO_3 solution prepared by dissolving 7.5 g of Na_2CO_3 in 92.5 mL of water was added. The vortexed mixture was incubated at room temperature for 2 hours and absorbance values were measured at 765 nm using a Thermospectronic Heliosα brand UV-Visible spectrophotometer. The experiments were repeated in triplicate and distilled water was used as blank. Being the blank absorbance A1 and the average absorbance of the three parallels A2,

absorbance differences (ΔA) were calculated from the following Equation 1.

$$\Delta A = A2\text{-}A1 \qquad (1)$$

The same procedure was repeated using seven standard gallic acid solutions with concentrations of 15.63, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. The absorbance differences were plotted against concentration to obtain a calibration graph. The total phenolic content of the extracts was given as mg gallic acid equivalent (GAE)/100 g air-dried sample.

The extracts were examined for DPPH radical scavenging antioxidant activities using the method of Cuendet et al. [13], with slight modifications. For this purpose, extracts with concentrations of 15.63, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL prepared from stock solutions were used (in some cases, samples with concentrations of 7.81 and 3.9 µg/mL were also added to the procedure). Aliquots of 750 µL of each of these extracts were mixed with 750 µL of the 1.10^{-4} M stable solution of the DPPH radical prepared in methanol, shaken vigorously in a vortex, and then incubated at room temperature for 50 minutes. Absorbances at 517 nm were measured using a Thermospectronic Heliosα brand UV-Vis spectrophotometer. Each sample was tested in duplicate and a tube containing only the sample solution and extract solvent of each concentration was used as a blank. Control tubes were prepared in triplicate and only DPPH· solution and its solvent (methanol) were placed in these tubes. After incubating for 50 minutes, their absorbances were measured at the same wavelength. The experiments were repeated using BHT as the reference compound. The means of the blanks were subtracted from the means of the absorbance values obtained. From these values, % scavenging (% I) values were calculated using Equation 2.

$$\%\ of\ DPPH\cdot\ Radical\ Scavenging\ Activity\ (I\%) = \frac{A_0 - A_1}{A_0} x100 \qquad (2)$$

where A_0 is the mean of the absorbance values of the control solutions (containing only DPPH· solutions), A_1 is the mean of absorbance values of sample tubes (containing extract and DPPH· solutions). The scavenging percentages were plotted against the concentrations of extract and reference compounds used. From these graphs, the concentration values (SC_{50}) of the extract and reference solutions, which reduced the I% values of the control tubes by half (50%), were calculated. Results are given in µg/mL.

The antioxidant activities of the extracts were also determined according to the cupric ion reducing antioxidant capacity (CUPRAC) method. For this purpose, the method of Apak et al. [14] was applied. After taking 1 mL of Cu-II solution, 1 mL of Neocuproin solution and 1 mL of NH_4Ac buffer solution (pH = 7) and mixing with X mL of antioxidant (or standard) solution, the total volume of the sample was adjusted to 4.1 mL by adding (1,1-X) ml of distilled water. After 1 hour of incubation, absorbance values were measured against the reagent blank at 450 nm using the same spectrophotometer mentioned above. The same procedures were repeated by preparing standard Trolox solutions with concentrations of 15.63, 31.25, 62.5, 125, 250, 500 µg/mL. From the values obtained, a calibration graph was prepared and CUPRAC values of the original extracts were calculated and given as g Trolox equivalent antioxidant capacity per 100 g of air-dried sample (g TEAC/100 g air-dried sample).

2.4. Statistical analysis

All experiments were performed in triplicate and the results are given as mean ± standard deviation (SD). Differences between the means were determined using statistical tests such as Kruskal Wallis, one-way analysis of variance (ANOVA). Tukey's Honestly Significant Differences (HSD) Post-Hoc test was used at a $p \leq 0.05$ significance level to explain the differences between the mean values. All statistical processes were performed using IBM SPSS Statistics for Windows (v. 20.0, IBM Corp., Armonk, NY, USA).

3. Results and discussions

3.1. Results of extraction yields

The yield values obtained from all three extraction techniques are given in Fig. 2 below in a comparative manner.

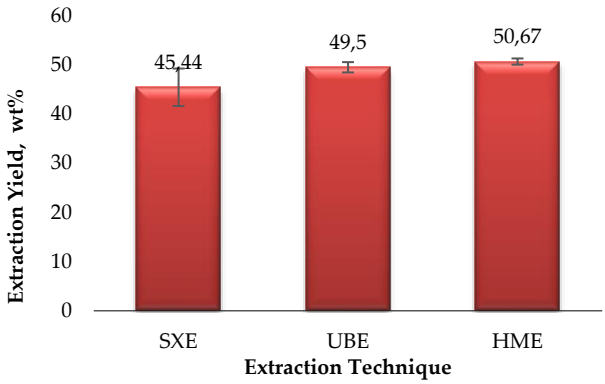

Figure 2. Extraction yield percentages obtained from the extraction techniques used (SXE: Soxhlet extraction, UBE: Ultrasonic bath extraction, HME: Hot methanol extraction)

As shown in Fig. 2, the highest extraction yield was obtained from HME. These values are 45.44 ± 3.81% for Soxhlet extraction, 49.50 ± 1.05% for ultrasound bath

extraction, and 50.67 ± 0.63% for hot methanol extraction, respectively. The Kruskal Wallis test, which is a nonparametric statistical test, was used instead of parametric one-way ANOVA since the data did not have in-group homogeneity ($p < 0.05$). Statistical treatment revealed that there were statistically significant differences between the extraction yields in terms of extraction techniques ($p < 0.05$). The yield of Soxhlet extractions was significantly lower than those of UBE and HME. The lower yield of the Soxhlet extraction compared to the other two techniques is thought to be due to the mass transfer problems between the solvent and the solid. Unless there is an external cause showing resistance, it is thought that the limiting step in the extraction of phenolic compounds from food samples is the diffusivities of the solutes from the solid to the solution [16]. Petrović et al. [17] showed that temperature and ultrasound positively affect mass transfer rate in slow extraction processes. Thus, considering the structure of the sample, it can be said that the transition of solutes from the matrix to the solution is more problematic in the Soxhlet technique compared to the other two techniques. Because the conditions in both UBE and HME techniques are harsher than in the SXE technique.

3.2. Results of TPC assay

The results obtained from the experiments performed using Folin Ciocalteu's Method are given in Fig. 3.

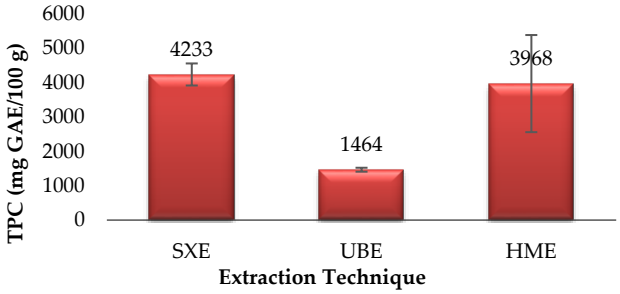

Figure 3. Total phenolic contents of the extracts (SXE: Soxhlet extraction, UBE: Ultrasonic bath extraction, HME: Hot methanol extraction)

The highest phenolic content in the experiments was obtained from SXE (4233 ± 321 mg GAE/100 g original sample) and HME (3968 ± 1412 mg GAE/100 g original sample) extracts, respectively ($p > 0.05$). The lowest TPC value was obtained in ultrasound-assisted extraction (1464 ± 57 mg GAE/100 g original sample). Of these three techniques, ultrasound-assisted extraction yielded significantly lower results than those of the other two techniques ($p < 0.05$). It is thought that this may be due to the fact that most of the compound groups that are extracted into the solution medium in ultrasound-assisted extraction do not have phenolic character. In the literature, many studies can be found showing that UBE is more advantageous than traditional extraction techniques in the extraction of phenolic compounds [18]. However, it should be noted that due to the complex nature of the sample structure and the diverse effects of phenolic compounds, there is no single and standard extraction method that can be applied to all herbal sample types at any time to extract their phenolic contents [19]. In addition, many variables such as device type (bath/probe), application power, frequency, temperature, solvent-solvent ratio, time, sample pretreatment should be considered, which affect the ultrasonic extraction process [20].

3.3. Results of antioxidant activity assays

3.3.1. Results of DPPH radical scavenging activity assay

Comparative SC_{50} values (mg/mL) obtained from DPPH radical scavenging antioxidant activity experiments are given in Fig. 4.

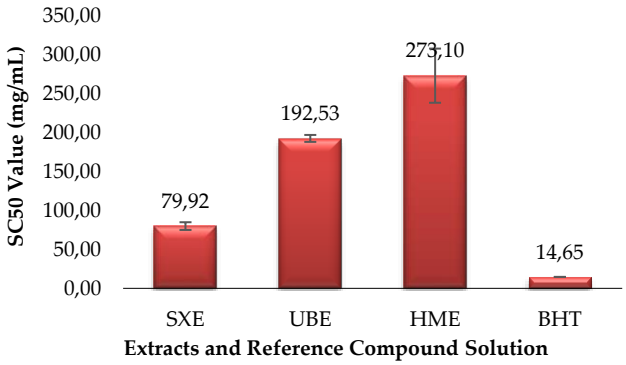

Figure 4. SC_{50} values of the extracts (SXE: Soxhlet extraction, UBE: Ultrasonic bath extraction, HME: Hot methanol extraction, BHT: Butylated hydroxytoluene) (Low value is better).

There were significant differences between the groups ($p < 0.05$) according to the nonparametric Kruskal-Wallis test performed because there was not any in-group homogeneity. In these experiments, the highest antioxidant activity was observed in Soxhlet extracts. The lowest values were obtained from hot methanol extracts. Values of ultrasonic bath extracts were between these two values (79.92 ± 4.96; 273.10 ± 34.79 and 192.53 ± 4.45 mg/mL, respectively). However, compared with BHT, all three extract types exhibited weaker antioxidant effects. For BHT, this value was obtained as 14.65 ± 0.08 mg/mL It is noteworthy that the antioxidant activity values of ultrasound-assisted extracts with lower phenolic content were higher than the antioxidant activity values of hot methanol extracts. This can be explained by the fact that in addition to the phenolic compounds, some other compound groups, which also have an antioxidant effect, pass into the solution in ultrasound-assisted extraction, or that some of the phenolic compounds that pass into the solution in hot methanol extraction do not show high antioxidant properties. Moreover, this reagent is not specific for

phenolic compounds, as it can be reduced with many non-phenolic compounds [21]. On the other hand, it can be said that some antioxidant compounds exposed to extreme conditions (high temperature and pressure) in the hot methanol extraction technique lose their antioxidant properties by breaking down or interacting with radical species formed under these conditions [22]. The DPPH radical scavenging antioxidant activity value measured in Soxhlet extracts of this plant is in agreement with the data in the literature [8].

3.3.2. Results of cupric ion reducing antioxidant capacity (CUPRAC) assay

The results obtained from the cupric ion reducing antioxidant capacity (CUPRAC) experiments are given in Fig. 5.

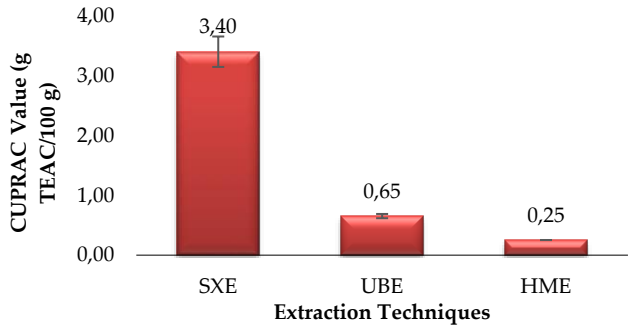

Figure 5. CUPRAC values of the extracts (SXE: Soxhlet extraction, UBE: Ultrasonic bath extraction, HME: Hot methanol extraction)

Although the non-parametric Kruskal-Wallis test showed that there was no significant difference between the CUPRAC values of the extracts obtained by three different methods ($p > 0.05$), it can be said that the Soxhlet technique revealed the best cupric ion reducing antioxidant capacity value (3.40 ± 0.25 g TEAC/100 g air-dried sample) according to the Mean Rank values. This value was followed by UBE (0.65 ± 0.04 g TEAC/100 g air-dried sample) and HME (0.25 ± 0.00 g TEAC/100 g air-dried sample), respectively. It can be easily seen that these results are in parallel with the results of the DPPH radical scavenging antioxidant activity method. Therefore, the comments made for the DPPH· method are also valid for this method.

In the literature, there are studies showing that high-pressure extraction processes are more successful than Soxhlet and ultrasound-assisted extraction processes in obtaining extracts with high total phenolic content [23, 24]. The results obtained in the present study also confirm these data. When it comes to the antioxidant properties of the extracts obtained, however, it is seen that different results are obtained. In one study, it was stated that the extracts obtained by high-pressure extraction showed higher antioxidant activity than the extracts obtained by ultrasound-assisted extraction technique [23]. However, in another study, it was reported that there was no difference between this technique and the other two techniques (Soxhlet and ultrasound-assisted extraction) compared with this technique [24]. A detailed literature search will reveal that quite different results are obtained for the extractions of similar compounds. It can be said that this variability is mainly related to the sample matrix [19], but besides this, the effect of some other variables such as extraction time, temperature, solvent type, and ratio, applied power should be considered [20].

It is an interesting research topic how the amount of phenolic content in a plant matrix affects the antioxidant activity of the extract obtained from that plant. While some researchers state that there is a strong linear correlation between them [25], others state that there is no such linear correlation or there is a more complex relationship than stated [24, 26]. Our study also revealed results that support this second group, that is, a positive linear correlation could not be established between TPCs and antioxidant activity values (both DPPH and CUPRAC).

4. Conclusions

In this study, dried fruits of the *D. Lotus* L. plant were extracted using three different extraction techniques such as Soxhlet, ultrasonic bath, and hot solvent (methanol) extraction, and their extraction yields and antioxidant activities were determined using two different methods (DPPH· and CUPRAC). Although the HME technique showed the highest extraction yield, it showed the same value as Soxhlet in terms of total phenolic content. UBE with the second-highest yield value revealed the lowest TPC value. In these respects, the HME technique seems to be competitive with the Soxhlet technique as it reaches a better extraction yield and the same TPC values with much less solvent consumption in a shorter time.

In terms of antioxidant activity values, the HME technique, which had the highest extraction efficiency and total phenolic content, revealed the lowest values. While the Soxhlet extraction technique showed the highest antioxidant activity values, interestingly the extract obtained from the UBE technique, which had the lowest TPC value, exhibited a higher antioxidant activity value than the extract obtained from the HME technique. This was attributed to the fact that there may be other components that can show antioxidant activity in the extract obtained from the UBE technique, apart from the phenolic components, or that the phenolic components in the extract obtained from the HME technique may be chemical species that do not have high antioxidant activity. However, more detailed analyzes are needed to

fully understand the reason for this phenomenon. Especially in UBE and HME techniques, it should be examined how the antioxidant activity values change depending on TPC values with the changing temperature and extraction times. However, it can still be said that the UBE technique has an advantage compared to the other two techniques due to its simplicity of application and the extraction of thermally sensitive compounds.

As a result, it can be said that the HME technique stands out with its advantages such as low solvent consumption and shorter extraction time in obtaining a phenolic-rich extract from the dried fruits of the *D. lotus* L plant, and the UBE technique is advantageous in terms of ease of application, shortened extraction time and protection of thermally sensitive compounds.

Acknowledgments

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Chemometric approach based on factorial and Box-Behnken designs for determination of anti-coronavirus drug; favipiravir in bulk and spiked human plasma by green HPLC method

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Abstract

The present work describes the development of a robust, sensitive, and green HPLC method with fluorescence detection for the determination of favipiravir (FAV). A fractional factorial design was implemented for the screening of different factors affecting chromatographic responses. The Box-Behnken design was applied to study and optimize the most critical method parameters. The optimum chromatographic conditions obtained involved the use of 0.1% phosphoric acid solution and isopropanol in the ratio 98:2 % v/v as mobile phase at a flow rate of 0.8 mL/min and column oven temperature of 35°C. Chromatographic analysis was performed on Eclipse plus® C18 (100 mm × 4.6 mm × 3.5 µm) column with fluorescence detector set at 361 nm and 432 nm for excitation and emission, respectively. A linear response was obtained over the range of 20 - 240 ng/mL with a limit of detection of 2.01 ng/mL and a quantitation limit of 6.11 ng/mL. The method was successfully implemented for the determination of FAV in its pharmaceutical formulation with a mean % recovery ± SD of 99.42 ± 0.59. Moreover, the sensitivity of the method allowed the determination of FAV in spiked human plasma over a range of 40-240 ng/mL. The combined application of green chemistry and quality by design leads to the development of a robust green method.

Keywords: Favipiravir, COVID-19 virus, Box-Behnken design, green chemistry, human plasma

1. Introduction

In December 2019, the first cases infected with the COVID-19 virus have emerged in Wuhan, China. Now, this virus becomes a pandemic all over the world. As of May 20, 2021, 33142485 cases have been diagnosed worldwide, and 444535 have died from the pandemic. COVID-2019 virus (also known as SARS-Cov-2) is a beta coronavirus which is enveloped positive strand RNA virus [1]. Unfortunately, no specific therapeutic agent has been approved for the treatment of the COVID-2019 virus till now. However, a number of already existing antiviral drugs which have been proved to be safe and effective against other viruses are tested for their activity against the COVID-19 virus. RNA-dependent RNA polymerase (RdRp) inhibitors are important. Favipiravir (FAV) which is known as T-705 is one of these drugs. Its chemical name is 6-fluoro-3-oxo-3,4- dihydropyrazine -2-carboxamide as shown in Fig. 1.

The Fujifilm Toyama Chemical Company has developed Favipiravir (Trade name: Avigan®) in Japan. It selectively inhibits RNA-dependent RNA polymerase (RdRP), an enzyme required for RNA viral replication inside human cells [2]. In 2020, after the COVID-19 pandemic has spread, different studies were carried out to evaluate the efficiency of FAV against coronavirus [3-6].

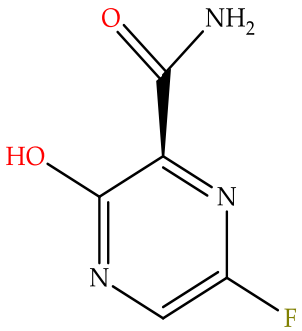

Figure 1. Chemical structure of favipiravir (FAV)

Literature review reveals that the reported methods for the analysis of FAV are HPLC with UV detection [7,8]. They involve the use of acetonitrile and phosphate solution as mobile phase. The spectrofluorimetric

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method has been developed [9]. No chromatographic method with fluorimetric detection has been developed for quantitation of FAV neither in pure form nor in pharmaceutical preparation.

Recently, there is increasing interest in the development of green analytical methods that replace the highly toxic solvents with more eco-friendly ones and reduce the number of organic solvents used. Different tools have been proposed for the assessment of analytical procedures greenness. Among those tools, two quantitative tools are available including analytical eco-scale, and HPLC-EAT (environmental assessment tool). Analytical eco-scale assesses greenness based on penalty points given for each reagent amount and type, the energy consumed by the electrical instruments, and the way of waste treatment [10]. HPLC-EAT developed by Gaber et al is a specific tool used for the assessment of chromatographic methods regarding mobile phase solvents [11].

Implementation of Quality by Design (QbD) principles in different pharmaceutical fields is gaining interest nowadays. The application of QbD principles to analytical method development focuses on the concept of building quality into the method during development, instead of testing methods for quality after development. Application of the QbD approach ensures a robust method by following a systemic and organized path in method development.

Some experimental designs have been employed for the development, optimization, and validation of various analytical techniques [12-17]. Box Behnken Design (BBD) is one of them that are employed to obtain optimum conditions by examining the factors at three levels with a fewer number of experimental runs compared to other three-level designs such as three-level factorial design and Central Composite Design (CCD)[18].

The aim of the current work was to develop a green, simple, rapid, and robust HPLC method for the quantitation of FAV in pure form, pharmaceutical formulation, and spiked human plasma. The current study involves the implementation of QbD principles for the development of a green chromatographic method for the quantitation of FAV. The green character is built in the method development from the first step by taking analytical eco-scale and EAT scores as Critical Quality Attributes (CQAs) during screening and optimization phases. The developed method is the first chromatographic method for the determination of FAV in plasma. It was compared to the reported one and found to be greener, faster, and more sensitive [7].

2. Experimental

2.1. Apparatus and software

Chromatographic separation was made using of Agilent Technologies 1260 Infinity instrument (Santa Clara, USA) equipped with a G1311C quaternary pump, G1329B autosampler, G13b16A thermostatic column compartment, G1314F UV variable wavelength detector (VWD) detector, and G1321C fluorescence detector (FLD). This instrument was linked to a Dell-compatible PC, bundled with Agilent OpenLAB CDS Chemstation Edition software. pH measurements were carried out by using HANNA pH 211 Microprocessor pH meter with a double junction glass electrode. Design Expert® (Version 11.1.2.0, Stat-Ease Inc. Minneapolis, MN, USA) software was employed to form and analyze both fractional factorial and Box Behnken designs.

2.2. Material and reagents

Analytical grade chemicals and reagents were used throughout the study. Favipiravir (FAV) powder (percent assay 100.1%) was obtained from Biophore India Pharmaceuticals Private Limited (Telangana, India). Analytical grade orthophosphoric acid purchased from Ridel-de Haën (Germany) was used. HPLC grade solvents as ethanol (Fisher Scientific, Germany), and isopropanol (Hipersolv, BDH Laboratory supplies, England) were used. All excipients used in the preparation of tablets were kindly supplied by the Pharmaceutical Technology Department, Faculty of Pharmacy, Tanta University (Tanta, Egypt). Human plasma was graciously provided by Tanta Blood Bank-Tanta international educational hospital (Tanta, Egypt). The study was approved by the Research Ethics Committee at Faculty of Pharmacy, Tanta University, Egypt (Protocol code: TP/RE/6-19PH-003).

2.3. Chromatographic conditions

Separation and quantitation were carried out on Eclipse plus® C18 (100 mm × 4.6mm × 3.5 µm) column using a mixture of water containing 0.1% phosphoric acid solution and isopropanol in a ratio of 98:2 v/v as mobile phase at a flow rate of 0.8 mL/min. The mobile phase was filtered through a 0.22 µm nylon membrane filter (Millipore, Ireland) before use. The column temperature was set at 35°C. The injection volume was 20 µL. The fluorescence detector was set at 361 nm and 432 nm for excitation and emission, respectively.

2.4. Standard solution

The stock solution of FAV was made in distilled water at a concentration of 400 µg/mL by dissolving an accurately weighed 10 mg of FAV in a calibrated 25-mL volumetric flask and completing the volume with water. The stock

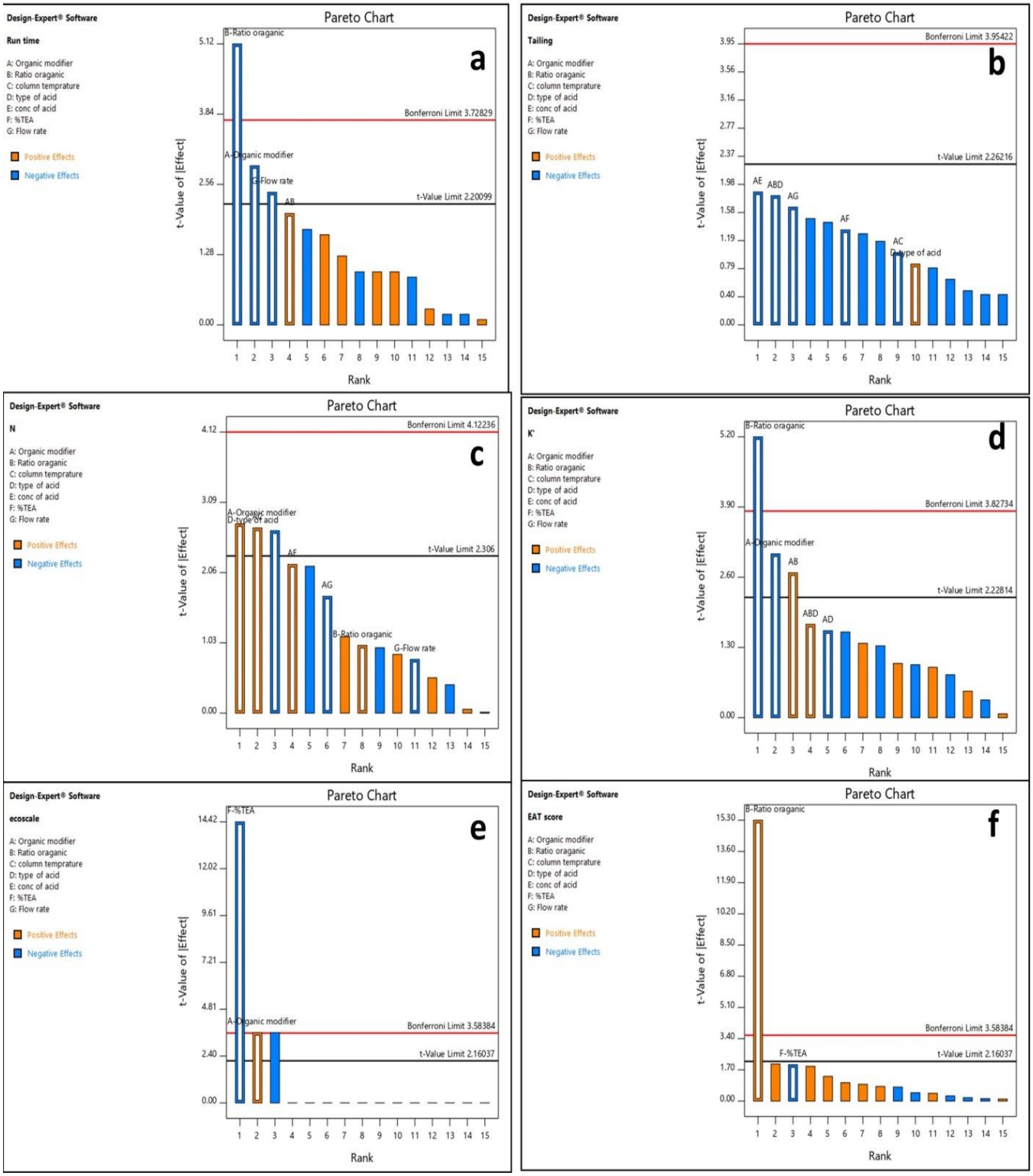

Figure 2. Pareto charts of the effects on the chromatographic responses: (a) run time, (b) tailing of FAV, (c) number of theoretical plates N, (d) capacity factor k', (e) analytical eco-scale score, and (f) EAT score

solution was stable in a refrigerator at 4°C for two weeks (based on 98% recovery). Working standard solution was prepared by taking an aliquot of 50 µL from the stock solution and diluting it to 25 mL with water in a calibrated 25-mL volumetric flask. The concentration of this working solution was 800 ng/mL.

2.5. Construction of the calibration curve

Into a set of 10-mL volumetric flasks, aliquots of FAV working standard solution were quantitatively transferred and diluted to 10 mL with mobile phase and mixed thoroughly to yield final concentrations in the range of 20 - 240 ng/mL. 20.0 µL portions of each solution were injected in triplicates under the previously mentioned chromatographic conditions.

2.6. Analysis of laboratory prepared tablet

The tablet of favipiravir is not available yet in the local market. So, a laboratory-prepared mixture imitating this tablet dosage form (Avigan® 200) was prepared. The amounts of excipients were deduced from the handbook of pharmaceutical excipients [19]. The tablet was obtained by mixing 200 mg FAV with the following excipients: 2.35 mg colloidal silicon dioxide, 7.05 mg povidone, 11.75 mg low substituted hydroxypropyl cellulose, 11.75 mg cross povidone, 10 mg talc, 2.35 mg sodium stearyl fumarate, and 5 mg titanium dioxide. An accurately weighed amount of the laboratory synthetic tablet containing 10 mg of FAV was moved into a calibrated 25-mL volumetric flask and 10 mL distilled water was added, sonicated for 10 min, and completed to the specified volume using distilled water. Then, the solution was filtered through a 0.45 μm membrane nylon filter. The first portion of the filtrate was discarded and then 50 μL of the filtrate was moved to a calibrated 25-mL volumetric flask and completed to the mark with distilled water. Finally, 1 mL of this solution was moved to a 10-mL volumetric flask and diluted to the specified volume with distilled water to procure a solution containing 80 ng/mL. The same procedure was applied for another five portions of the synthetic tablets. The concentration of FAV in the tablet was determined by using the regression equation.

2.7. Application to human plasma

100 μL of plasma was transferred and spiked with FAV standard solutions 25-150 μg/mL so that FAV concentration in plasma lies within the range of 5-30 μg/mL. Then, 1 mL isopropanol was added to precipitate protein. Afterward, the mixtures were vortex-mixed for two minutes then centrifuged at 4000 rpm for 10 minutes. Then the supernatant was transferred into a 5-ml volumetric flask and completed to the mark with the mobile phase. For each mixture, an aliquot of 4 mL was transferred to a calibrated 10-mL volumetric flask, and the mobile phase was added up to the mark to obtain final concentrations in the range of 40-240 ng/mL. 20.0 μL portions of each solution were injected in triplicates under the previously mentioned chromatographic conditions. Blank experiments were made out by handling FAV free plasma samples in a similar way. The regression equation was derived.

$$y = 10.8387x + 120.9383$$

where y and x represent mean peak area and FAV concentration (ng/mL), respectively.

3. Results and Discussion

3.1. Method development and optimization

The quality by Design approach was used throughout method development. First, the goal of the method (analytical target profile) is defined. The goal was the development of a fast green-sensitive HPLC method for the determination of FAV. Then, CQAs are outlined including short analysis time, good peak symmetry, increased number of theoretical plates, capacity factor k', and greenness of the method. The greenness of the method was taken as a response to be optimized from the beginning to reach the optimum chromatographic conditions regarding analytical performance as well as greenness. After that, CMPs which could have a possible impact on CQAs are identified from preliminary experiments.

3.1.1. Screening of parameters

Preliminary experiments were first performed to find out the most suitable column and composition of the aqueous portion of the mobile phase. Different columns were tried including X-Bridge™ (150 mm x 4.6 mm, 5 μm) column, Inertsil ODS-3(250 mm x 4.6 mm, 5 μm) column, and Eclipse plus® C18 (100 mm × 4.6mm × 3.5 μm) column. The latter was found to be the best column regarding run time and peak shape. Different buffers were tried including phosphate and acetate buffers, but the peak was forked and not symmetric. When phosphoric acid and acetic acid were tried, the accepted peak was obtained. So, the type of acid used, and its concentration were further studied. A fractional factorial design was applied for the screening of parameters because it is the most suitable design for the evaluation of a large number of factors and detecting interaction between these factors. A regular (2^{7-3}) two-level fractional factorial design (Res IV) was selected for the screening of seven factors which are the type of acid, concentration of acid, organic modifier, the ratio of organic modifier, triethylamine concentration, column temperature, and flow rate. The low and high levels of each factor were determined. A set of 16 experiments were performed as shown in the design matrix in the supplementary material (Table S1). The responses obtained for CQAs (tailing of the peak, run time, number of theoretical plates N, capacity factor k', analytical eco-scale, and EAT score) were recorded. The greenness of the method was taken into account as one of the CQAs needs to be studied during the screening phase, in the form of analytical eco-scale and EAT score. Analytical eco-scale alone is not enough as an indication for the greenness because small changes in the volume used of organic solvents are not adequately reflected. So, EAT

score was used along with eco-scale as responses reflecting method greenness.

3.1.2. Fractional Factorial Design results

Pareto charts were used to identify the most statistically significant factors that have an impact on different responses. Factors that exceed the t-limit line are significant. Pareto charts in Fig. 2 show that the ratio of organic modifiers has a statistically significant effect on run time, capacity factor, and EAT score while flow rate strongly affects run time. Isopropanol was found to be better than ethanol regarding run time and method greenness. Triethylamine has a negative effect on the eco-scale score while it does not significantly affect any other CQA, so it was excluded from the mobile phase to improve method greenness. Although column temperature was not found to be statistically significant for any CQA, the interaction between column temperature and organic modifier has a significant effect on the number of theoretical plates, so it was further studied and optimized. Based on the results of Pareto charts, three parameters that have the greatest significance were selected to be optimized including the ratio of organic modifier, flow rate, and column temperature. The other four factors were kept at the value that gives better chromatographic performance and greenness (organic modifier isopropanol, 0.1% phosphoric acid, without triethylamine).

3.1.3. Optimization using Box Behnken Design

For optimization, the Box Behnken design was selected because it gives a quadratic model with a fewer number of experimental runs compared to Central Composite Design (CCD). A set of 15 experiments were carried out and responses were recorded as shown in the supplementary material (Table S2). For all experimental runs in Box Behnken optimization, the Analytical eco-scale is constant (equals 90) because isopropanol is the organic modifier in all experiments. So eco-scale was excluded from responses to be optimized because it does not change with small changes in the volume of organic solvent, unlike EAT score.

3.1.4. Box Behnken design Results

The design expert software analyzed the data and generated different plots that helped to understand the impact of different factors such as interaction plots, contour plots, and response surface plots. The three-dimensional surface plots shown in Fig. 3 illustrate the effects of two factors on the response at a time. Optimum chromatographic conditions were reached using a desirability plot in the supplementary material (Fig. S1) which involves the use of 0.1% phosphoric acid solution and isopropanol in the ratio 98:2 % v/v as mobile phase at a flow rate of 0.80 mL/min and column oven temperature of 35°C. HPLC Chromatogram of 80ng/mL of FAV under the optimum conditions is shown in Fig. 4. Results for system suitability tests of the proposed HPLC method are listed in the supplementary material (Table S3).

3.1.5. Development of Design Space

The design space in the QbD-approach describes the whole range of interactions between critical method parameters (CMPs) and their effects on CQAs that have been examined during the optimization process. This approach provides assurance on the quality of the developed method. Regions in which the specifications of a chromatographic response are not met are shaded out, leaving the yellow area which is a favorable region as shown in the supplementary material (Fig. S2). This means that in this design space, i.e., the yellow region, the method is robust and changes made in this region would not affect the quality of the proposed method.

3.2. Method validation

The method validation was carried out following the guidelines of ICH [20]. Limit of detection (LOD), the limit of quantitation (LOQ), linearity and range, accuracy, precision, and robustness were investigated.

3.2.1. Linearity and range

The linearity was confirmed by the construction of the calibration curves by plotting the mean peak area of FAV, on the y-axis, against the corresponding concentration of FAV in ng/mL, on the x-axis. Statistical results were given in Table 1.

Table 1. Linearity regression data for FAV in bulk using the proposed HPLC method.

Parameter	Value
Linearity range (ng/mL)	20 - 240
slope	17.04
SE of slope	0.12
Intercept	-34.04
SE of Intercept	16.63
Correlation coefficient (r)	0.9997
SE of estimation	26.05

3.2.2. LOQ and LOD

The guidelines of ICH (Q2 (R1)) were followed for the estimation of LOQ and LOD based on the standard deviation of the blank response and the slope of the calibration curve. The found values of LOD and LOQ were 2.01 and 6.11 ng/mL respectively. These values indicate the high sensitivity of the proposed chromatographic method.

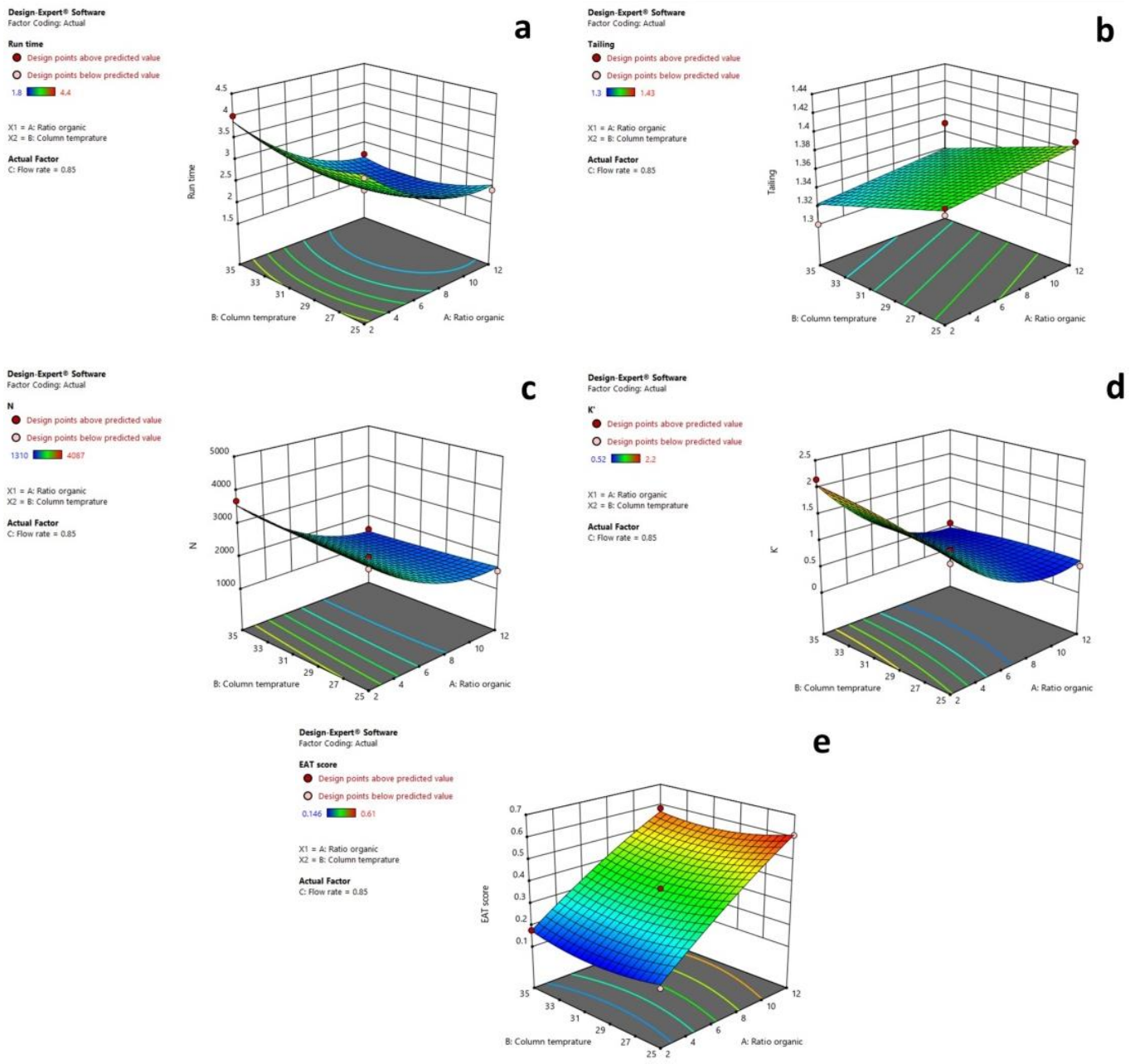

Figure 3. Response surface plots showing the impact of factors on the chromatographic responses: (a) run time, (b) tailing of FAV, (c) number of theoretical plates N, (d) capacity factor k', and (e) EAT score

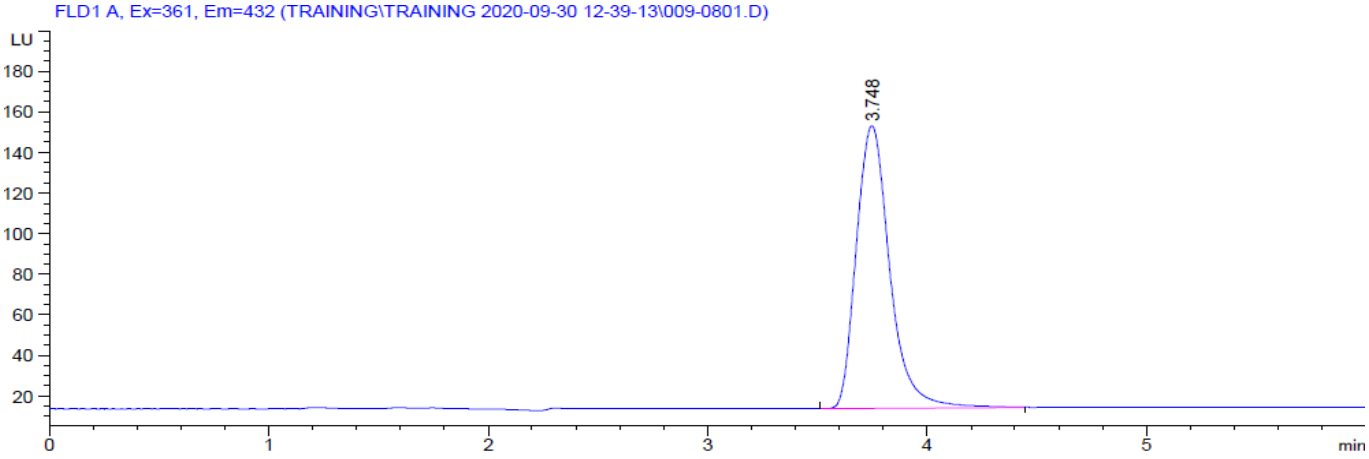

Figure 4. HPLC Chromatogram of 80 ng/mL of favipiravir (FAV) using a mixture of 0.1% phosphoric acid solution and isopropanol in the ratio 98:2 % v/v as mobile phase

Table 2. Evaluation of accuracy for the determination of FAV using the proposed HPLC method

Drug	Conc. taken (ng/mL)	Conc. found (ng/mL)*	% Recovery	Mean % recovery ± SD
FAV	60	59.92	99.86	99.42 ± 0.59
	80	79.00	98.75	
	100	99.65	99.65	

*** mean of three determinations.**

3.2.3. *Accuracy and precision*

The accuracy of the proposed method was evaluated by performing the general analytical procedure for estimating three solutions containing different concentrations of FAV within the linearity range. The measurement was carried out in three replicates and the recovery percentage and standard deviation (SD) were determined. The % recovery values were close to 100% giving a sign for the respected degree of accuracy of the suggested method (Table 2). Triplicate samples corresponding to three different concentrations of FAV were assayed in one day and in three different successive days, respectively, to determine intra and inter-day precisions. The small values of % RSD were significant for the precision of the method (Table 3).

3.2.4. *Robustness*

The robustness of the current methodology was investigated by introducing small yet deliberate variations within its optimized parameters. The examined parameters in the robustness testing were the change in isopropanol ratio (2% ± 1), column temperature (35°C ± 2), and flow rate (0.8 mL/min ± 0.1). The relative standard deviation was still less than 2% indicating the robustness of the method as shown in the supplementary material (Table S4).

Table 3. Application of the proposed HPLC method and comparison method [5] for the determination of the FAV in laboratory prepared tablet

Drug	Intra day			Inter day		
	conc. taken (ng/mL)	conc. found (ng/mL)	%RSD	conc. taken (ng/mL)	conc. found (ng/mL)	%RSD
FAV		60.15			59.72	
	60	59.86	0.85	60	59.05	0.76
		59.16			58.87	
		78.54			79.00	
	80	79.00	0.58	80	77.65	0.96
		79.46			77.77	
		99.05			99.65	
	100	99.42	0.74	100	99.23	0.67
		100.47			98.34	

3.3. Applications

3.3.1. *Analysis of the pharmaceutical formulation*

The proposed method was applied for the analysis of FAV in its laboratory-prepared tablet with no interference from excipients. The percent recovery ± S.D. was 98.97 ± 0.76 indicating the high accuracy and precision of the method. The chromatogram of FAV in the synthetic mixture is shown in the supplementary material (Fig. S3). The percent recovery values of the proposed method were compared with that of the reference method (5). There was no significant difference between the developed method and the comparison method as indicated by statistical analysis of the results using student's t-test and the F-test (Table 4).

Table 4. Application of the proposed HPLC method and comparison method [5] for the determination of the FAV in laboratory prepared tablet

Sample No.	Proposed method% Recovery of FAV	Reported method% Recovery of FAV
1	99.62	98.25
2	100.06	97.26
3	98.60	99.45
4	98.24	100.26
5	99.36	101.02
6	97.96	99.23
Mean % recovery ± S.D.	98.97 ± 0.76	99.25 ± 1.24
t-test	0.683	(2.228)*
F-test	2.647	(5.050)*

*Values in parenthesis are the tabulated t- and F- values at p=0.05

3.3.2. *Application in spiked human plasma*

Different studies were carried out to determine the pharmacokinetic effects of multiple doses of FAV. Cmax of FAV ranged from 22.01µg/mL to 36.24 µg/mL in healthy American and Japanese subjects [21]. The proposed HPLC method was investigated for its capability to determine FAV in plasma samples through a linear range from 40 to 240 ng/mL. Different solvents were tried to precipitate plasma proteins including methanol, ethanol, isopropanol, and acetonitrile. Isopropanol was selected as it gives the best percent extraction (91.54 %) with a good peak shape. The plasma calibration curve was linear with the following equation: $y = 10.8387 x + 120.9383$ (r^2=0.9985). The method has been carried out on blank plasma in order to check the effect of the interfering matrix as shown in the supplementary material (Fig. S4).

Table 5. Application of the proposed chromatographic method to the determination of FAV in spiked human plasma

Conc. added (ng/mL)	Mean conc. found (ng/mL)*	Mean % recovery ± SD	%RSD
40	38.24	95.59 ± 4.23	4.43
80	78.17	97.71 ± 1.99	2.04
120	123.12	102.60 ± 2.54	2.48
160	163.38	102.12 ± 2.11	2.06
240	237.09	98.79 ± 3.45	3.49

The selectivity of the proposed method was proven by the determination of FAV in spiked human plasma samples without interference from the endogenous

matrix. The results revealed the suitability of the method for the assay of FAV in spiked human plasma with acceptable recovery (Table 5).

3.4. Assessment of the method greenness

The greenness of the proposed chromatographic method was proved by calculating the analytical eco-scale and EAT scores. The calculated eco-scale was found to be 90 as shown in the supplementary material (Table S5). The proposed method was compared to the reported methods regarding sensitivity, greenness, and analysis time. Results shown in the supplementary material (Table S6) prove that the developed method is faster, greener, and more sensitive than the reported one.

4. Conclusion

The current study was carried out to develop a green-sensitive chromatographic method with fluorescence detection for rapid quantitation of the new antiviral drug; favipiravir that has a potential therapeutic effect against the COVID-19 virus. The quality by Design approach was implemented throughout method development. The combination of QbD and green analytical chemistry results in the development of a design space that provides assurance of quality and improves method robustness. The adopted method is simple, fast, robust, and green and can replace the reported HPLC methods for the determination of favipiravir in routine analysis. Moreover, the high sensitivity of the proposed method allowed the determination of FAV in spiked human plasma.

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Suplementary materials

Table S1. Fractional factorial design matrix for the effect of different CMPs on CQAs

Run	A:Organic modifier	B:Organic ratio	C:column temprature	D:type of acid	E:conc of acid	F:%TEA	G:Flow rate	Run time	Tailing	N	k'	ecoscale	EAT score
1	EtOH	5	40	phosphoric acid	0.1	0.01	1	2.3	1.34	1853	0.96	80	0.231
2	IPA	5	40	phosphoric acid	0.01	0.01	0.7	1.8	1.33	2117	0.45	82	0.164
3	EtOH	25	40	phosphoric acid	0.01	0	1	1.2	1.26	1644	0.28	88	0.604
4	IPA	5	40	acetic acid	0.01	0	1	1.4	1.42	1823	0.21	88	0.182
5	IPA	5	25	phosphoric acid	0.1	0	1	2.5	1.25	2082	1.16	90	0.325
6	EtOH	25	25	acetic acid	0.1	0	1	1.2	1.42	1498	0.26	86	0.604
7	IPA	25	40	acetic acid	0.1	0.01	1	1	0.97	2053	0.18	80	0.65
8	EtOH	25	25	phosphoric acid	0.1	0.01	0.7	1.6	1.33	1920	0.32	80	0.563
9	IPA	25	25	phosphoric acid	0.01	0.01	1	0.9	1.34	1489	0.24	82	0.585
10	IPA	25	25	acetic acid	0.01	0	0.7	1.3	1.45	1743	0.26	88	0.591
11	EtOH	5	25	acetic acid	0.01	0.01	1	2.4	1.35	1722	1	78	0.241
12	IPA	5	25	acetic acid	0.1	0.01	0.7	2.1	1.44	1764	0.2	80	0.191
13	EtOH	5	25	phosphoric acid	0.01	0	0.7	3.6	1.27	1896	1.16	88	0.254
14	EtOH	5	40	acetic acid	0.1	0	0.7	4.6	1.29	766	1.35	86	0.324
15	IPA	25	40	phosphoric acid	0.1	0	0.7	1.4	1.29	3538	0.26	90	0.637
16	EtOH	25	40	acetic acid	0.01	0.01	0.7	1.5	1.39	1501	0.23	78	0.528

TEA: Triethylamine, EtOH: ethanol, IPA: isopropyl alcohol, N: number of theoretical plates, k': capacity factor

Table S2. Box Behnken design matrix for the effect of different CMPs on CQAs

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Run	**A:Ratio organic**	**B:Column temprature**	**C:Flow rate**	**Run time**	**Tailing**	**N**	**K'**	**EAT score**
1	7	30	0.85	2.4	1.41	1940	0.81	0.371
2	7	35	1	2.2	1.31	1622	0.73	0.4
3	12	35	0.85	2.2	1.32	1548	0.56	0.583
4	12	30	0.7	2.5	1.43	1908	0.63	0.546
5	7	25	1	2.5	1.31	1754	0.75	0.455
6	2	30	1	2.8	1.38	2585	1.6	0.146
7	2	30	0.7	4.4	1.37	4087	2.2	0.16
8	2	25	0.85	3.6	1.37	3091	1.46	0.159
9	2	35	0.85	4	1.3	3694	2.15	0.177
10	12	25	0.85	2.3	1.39	1556	0.52	0.61
11	7	25	0.7	3.2	1.38	2374	0.89	0.407
12	7	35	0.7	3	1.34	2303	0.84	0.382
13	12	30	1	1.8	1.35	1310	0.66	0.561
14	7	30	0.85	2.4	1.31	1994	0.82	0.371
15	7	30	0.85	2.3	1.34	1962	0.83	0.356

Table S3. Results of system suitability tests for the proposed HPLC method

Parameter	**FAV**
Retention time (R_T) (min)	3.75
Capacity factor (k')	2.39
Theoretical plates (N)	3818
Asymmetry factor	1.34

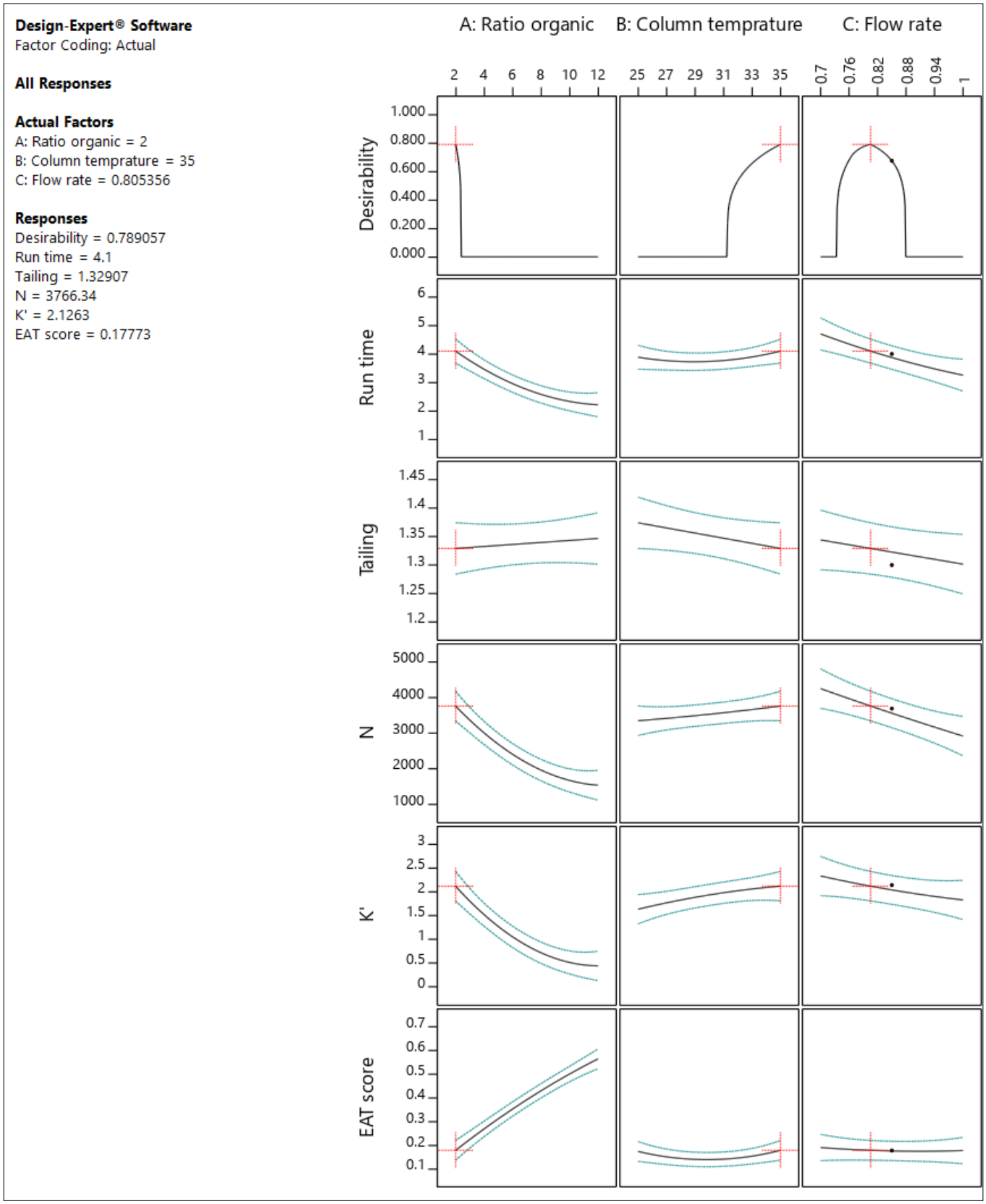

Figure S1. Desirability plot showing optimum chromatographic conditions

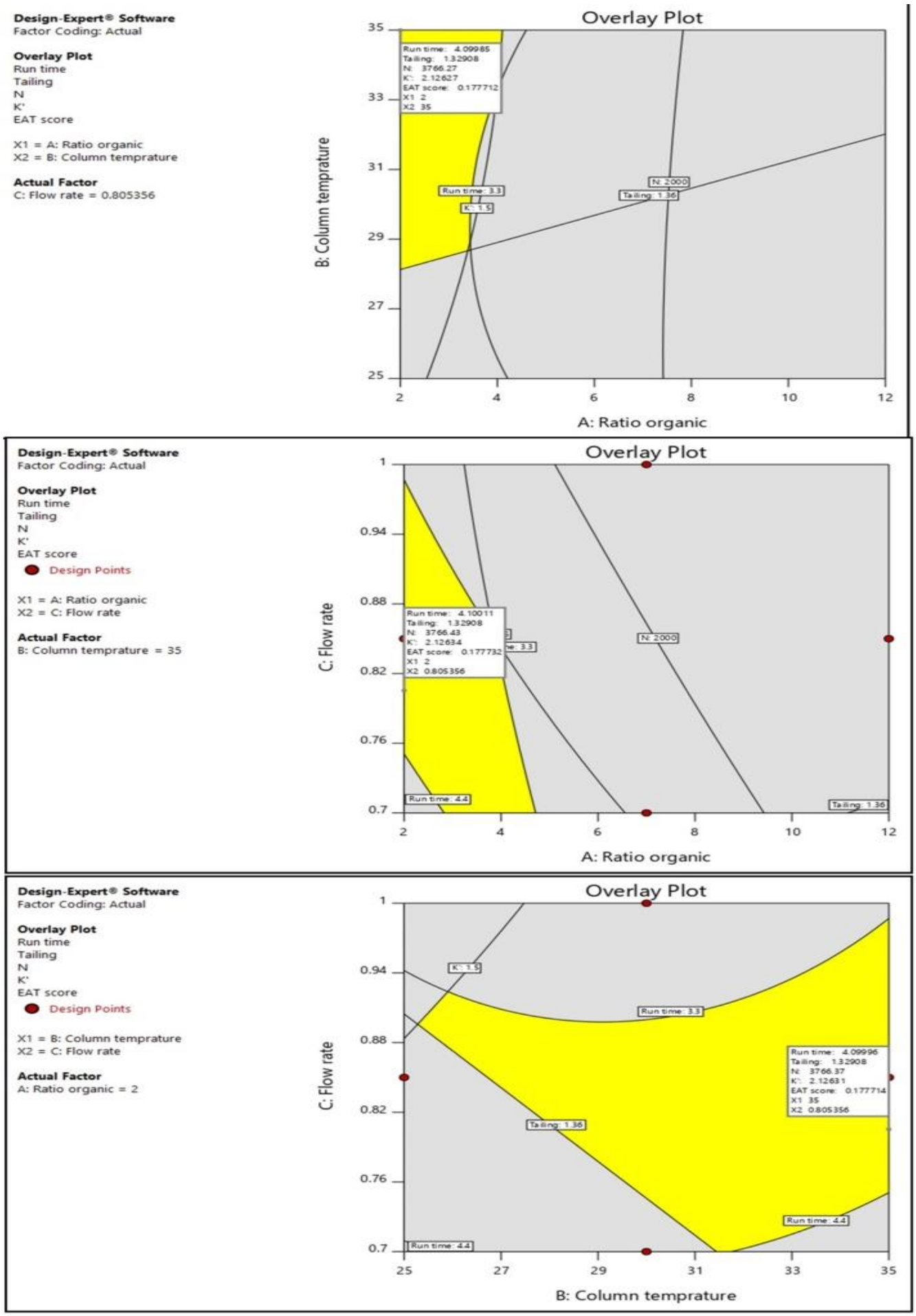

Figure S2. Design space (overlay plot) for different chromatographic conditions

Table S4. Robustness results for the proposed HPLC method

Parameters		FAV			
		Recovery %	Mean recovery %	S.D	R.S.D
Ratio of isopropanol (%)	1	97.72	98.72	0.80	0.81
	2	98.75			
	3	99.69			
Flow rate (mL/min)	0.7	98.52	98.92	0.41	0.41
	0.8	98.75			
	0.9	99.48			
Column temperature (°C)	33	100.13	99.16	0.69	0.69
	35	98.75			
	37	98.59			

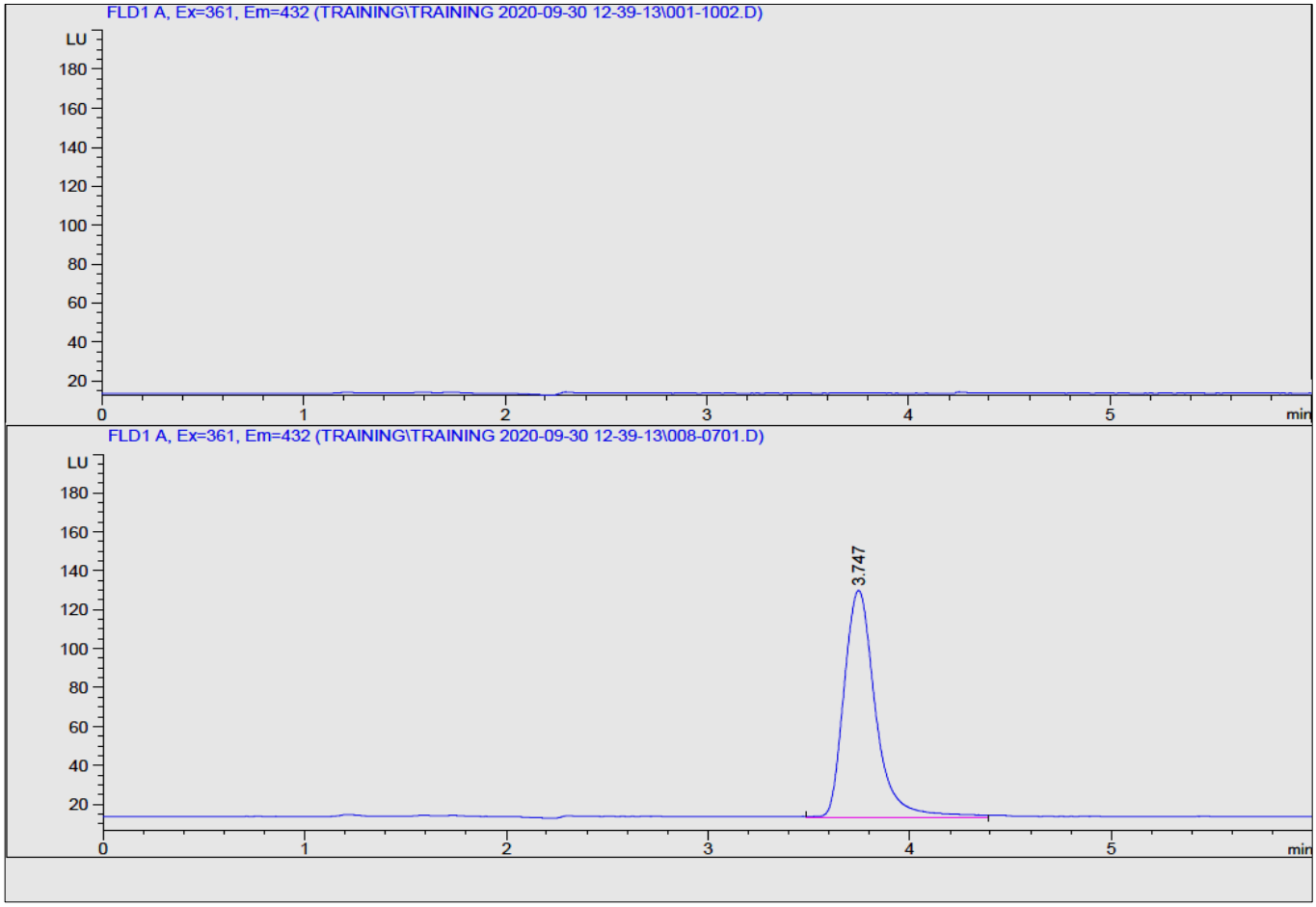

Figure S3. Chromatograms of (a) blank experiment containing all possible excipients, and (b) laboratory prepared mixture containing 80 ng/mL FAV using the proposed HPLC method

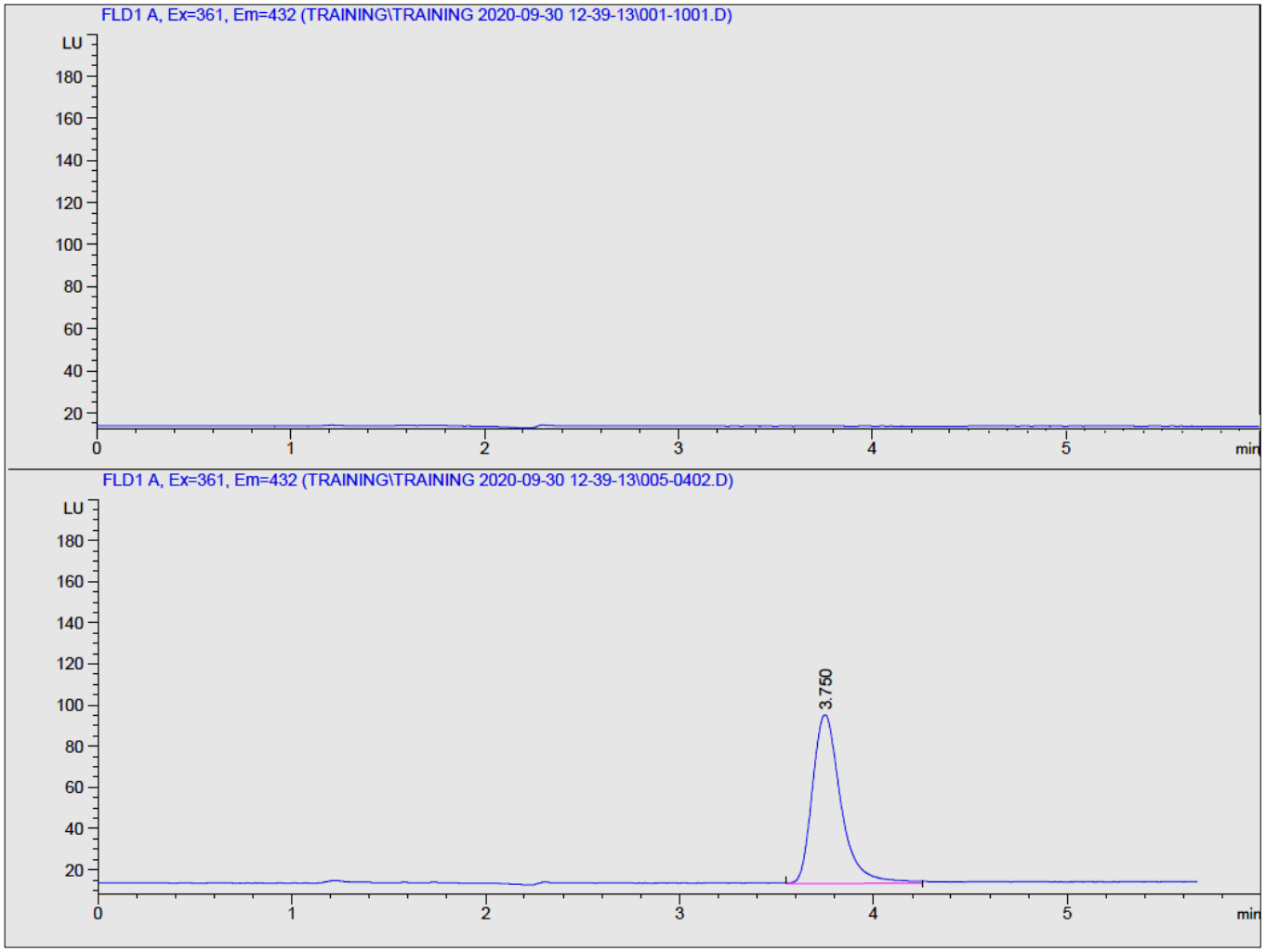

Figure S4. Chromatograms of (a) blank plasma sample, and (b) plasma sample containing 80 ng/mL FAV using the proposed HPLC method

Table S5. Calculation of analytical eco-scale for the developed method

Reagents/Instruments	**Penalty points**
Reagents	
Phosphoric acid	2
isopropanol	4
Instruments	
HPLC	1
Occupational hazards	0
Waste	3
Total penalty points	∑ 10
Analytical eco-scale score	90

Table S6. Comparison of the proposed HPLC method with the reported one

	Reported method [5]	**Reported method [6]**	**Proposed method**
Linearity range	2-8 µg/mL	10-100 µg/mL	40 - 240 ng/mL
Limit of detection (LOD)	NA	1.20 µg/mL	2.00
Limit of Quantitation (LOQ)	NA	3.60 µg/mL	6.11
Analysis time (min)	11.0	15.0	4.5
Analytical eco-scale score	83	83	90
HPLC-EAT score	3.934	5.364	0.187