Journal of Equine Veterinary Science 113 (2022) 103938

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Anti-Methanogenic Traits of Safflower Oil Compounds Against Methyl-Coenzyme M Reductase Receptor in Equines: An *In Silico* Docking Analysis

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ARTICLE INFO

Article history:
Received 1 March 2022
Received in revised form 18 March 2022
Accepted 21 March 2022
Available online 25 March 2022

Keywords:
Anti-methanogenic agent
Equines
In silico
Methanogenesis
Methane
Safflower oil

ABSTRACT

Greenhouse gases emission from livestock is the major concern for the ecosystem. Despite the lower contribution of non-ruminants towards greenhouse gas emission as compared to the ruminants, the emission of methane (CH_4) gas from equines is expected to be increased in future due to its increasing population. Thus, it is essential to find or screen potential anti-methanogenic agent in a cost-effective and quicker manner. Considering this, the present investigation was aimed to analyze anti-methanogenic characteristic of bioactive compounds of safflower oil by targeting methanogenesis catalyzing enzyme (Methyl-coenzyme M reductase; MCR) via *in silico* tool. Initially, a total of 25 compounds associated with safflower oil were selected and their drug-likeness traits were predicted through Lipinski's rule of 5. Of 25 compounds, 9 compounds passed all the parameters of Lipinski's rule of five. These 9 ligands were further submitted for ADME traits analysis using Swiss ADME tool. Results revealed the absence of Lipinski's violation and approval of drug-likeness attributes of methyl tetradecanoate, 3-isopropyl-6-methylenecyclohex-1-ene, trans-2,4-decadienal, cis-6-nonenal, limonene, syringic acids, matairesinol, acacetin, and 2,5-octanedione. Molecular docking analysis was performed for analyzing the affinity between the selected 9 ligands and MCR receptor using FRED v3.2.0 from OpenEye Scientific Software and Discovery Studio client v16.1.0. Results showed maximum binding interaction of acacetin with MCR with the chemguass4 score of -13.35. Other ligands showed comparatively lower binding affinity in the order of matairesinol (-12.43) > methyl tetradecanoate (-9.25) > cis-6-nonenal (-7.88) > syringic acids (-7.73) > limonene (-7.18) > trans-2,4-decadienal (-7.07) > 3-isopropyl-6-methylenecyclohex-1-ene (-7.01) > 2,5-octanedione (-7.0.). In a nutshell, these identified compounds were observed as potential agents to reduce CH_4 production from equines by targeting MCR. This *in silico* study emphasized the role of safflower-associated compounds in developing anti-methanogenic drug for equines in future.

Conflict of interest statement: The authors declare no conflicts of interest.
Animal welfare/ethical statement: Not applicable.

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https://doi.org/10.1016/j.jevs.2022.103938
0737-0806/

1. Introduction

At present, global warming is an irrefutable fact which is becoming a huge concern for the humankind. The emission of greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2) from livestock is the leading factor of global warming [1]. The emission of CH_4 is the most concerning because it shows approximately 30 times higher global warming potential than CO_2 [2]. Herbivores emit higher rate of CH_4 than other monogastric animals because they mainly consume fibrous forages. However, non-ruminants, especially horses emit 3.3-fold less CH_4 than ruminants. Nevertheless, in view of the escalating populace of equines per year, the emission of CH_4 from equines is expected to be increased in future [3].

Over the past few years, various strategies have been adopted to mitigate the emission of CH_4 from livestock [4–6]. Moreover, the supplementation of plants' leaves and its metabolites as feed additives has shown promising strategy towards the mitigation of CH_4 from equines, particularly horses [3]. In general, methanogens are known to produce CH_4 in the hind gut of horses by reducing CO_2. However, based on the substrates utilized or methanogenic pathways, methanogens are classified into 3 classes: (i) microbes utilizing CO_2 as substrate, (ii) microbes utilizing methyl group carbon attached to oxygen, nitrogen, and sulphur, and iii) microbes utilizing acetate as carbon source (Fig. 1) [7]. Methanogens require Methyl coenzyme M reductase (MCR; EC 2.8.4.1) for methanogenesis process. MCR is a dimer of heterotrimers with a molecular weight of about 300 kDa. It contains 3 subunits in an $(\alpha\beta\gamma)_2$ stoichiometry [8]. It also constitutes a catalytic active coenzyme F_{430} (nickel containing tetrapyrrole) as a prosthetic group tightly bound to each monomer. Thus, MCR is a marker of methanogenesis process [9].

Now a days, the computational simulations have shown immense potential in reducing the experimental costs. *In silico* docking analysis has escalated the drug discovery process efficiently by analyzing virtually the database of plethora of bioactive components [10–12]. In our previous *in vitro* investigation, we have successfully demonstrated the significant reduction of CH_4 emission from horses by utilizing safflower (*Carthamus tinctorius* L.) oil as a feed additive [6].

Safflower is a medicinal herb of Compositae family. It is a multi-purpose oil seed crop which is widely cultivated in Asia, Europe, Mexico, and Australia [13]. Seeds of safflower contain oil (30%), protein (20%), and crude fiber (35%). Safflower oil contains 70% polyunsaturated fatty acid (linoleic acid) and 10% monounsaturated (oleic acid) with small amounts of stearic acid [14]. It is often used as ideal feed for livestock due to the presence of polyunsaturated fatty acid in edible oil. The vast biological at-

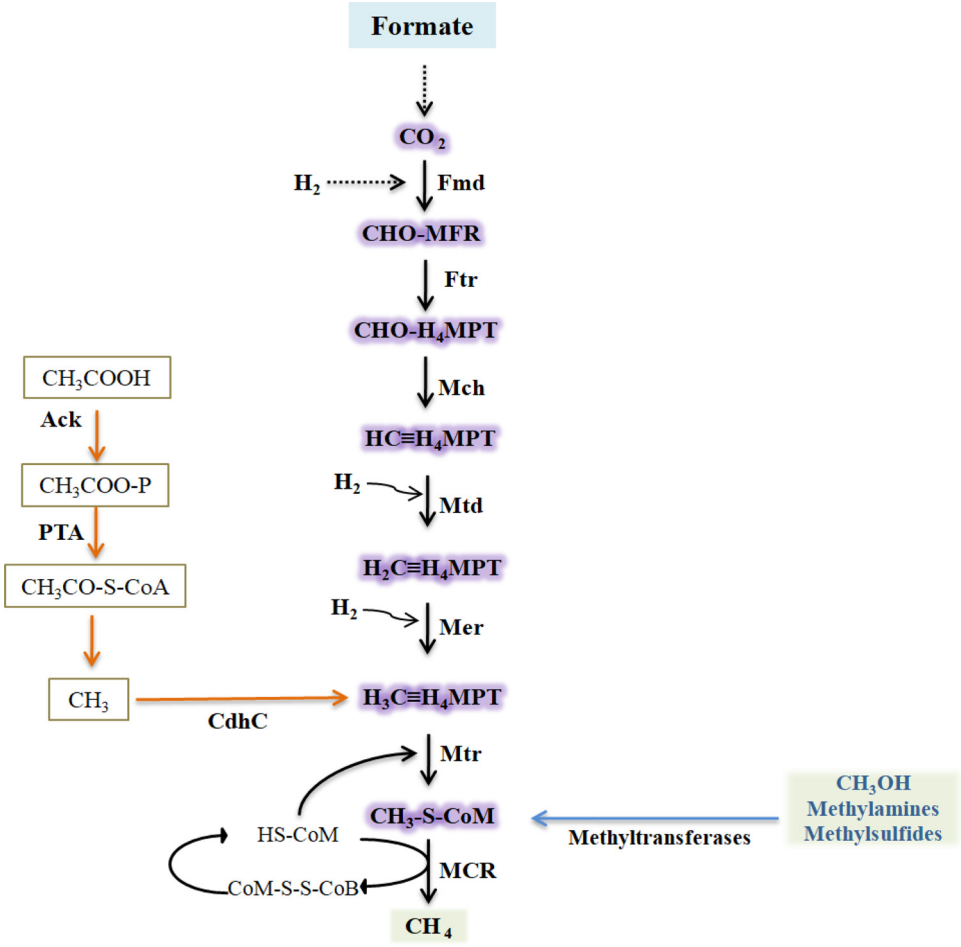


Fig. 1. Methanogenic pathways by methanogens using CO_2, methyl group compounds, and acetate as substrates.
(Fmd – Formylmethanofuran dehydrogenase; CHO-MFR – Formylmethanofuran; Ftr – Formylmethanofuran H_4MPT formyl transferase; Mch – Methenyl-cyclohydrolase; H_4MPT – Tetrahydromethanopterin; HC≡H_4MPT – Methylene-H_4MPT; Mtd – F_{420}-dependent methylene-H_4MPT dehydrogenase; H_3C-H_4MPT – Methyl-H_4MPT; Mer – Methylene-H_4MPT reductase; Mtr – Methyl-H_4MPT coenzyme M methyl transferase; CH_3-S-CoM – Methyl-coenzyme M; MCR – Methyl-coenzyme M reductase; CoM-S-S-CoB – Coenzyme M-coenzyme B heterodisulfide; Cdhc – CO dehydrogenase/acetyl-CoA synthase complex; Ack – Acetate kinase; PTA – Phosphate acetyl transferase).

tributes of this plant have represented safflower oil as a potential herb of interest for researchers [15].

However, it is essential to analyze the anti-methanogenic role of specific bioactive components of safflower oil as ideal supplement. Computational tools such as molecular docking can certainly help screening particular phytocomponents against target receptors and minimize the cost as well as prolonged duration of *in vitro* or *in vivo* experiments [9,16]. From this point of view, in this study, we have undertaken further a significant attempt to analyze the interaction of certain biologically-active compounds of safflower oil with MCR via *in silico* tools for suggesting CH_4 mitigating or anti-methanogenic role of safflower oil by targeting MCR.

2. Materials and Methods

2.1. Phytocompounds Used

Based on the previous reports revealing the presence of diverse compounds in safflower oil [13–15,17], we selected a total of 25 compounds in this investigation as shown in Fig. 2.

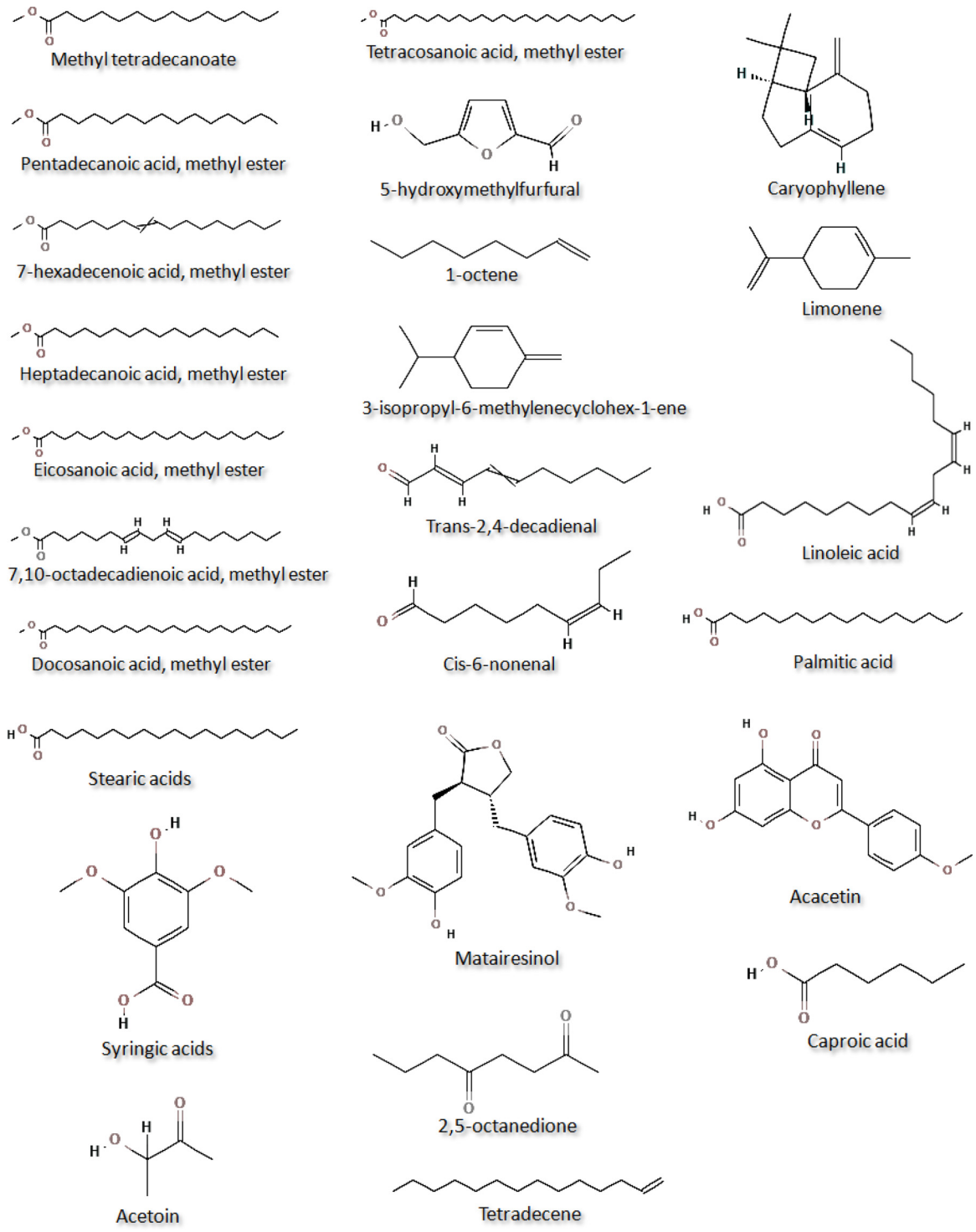


Fig. 2. Structure of safflower oil compounds.

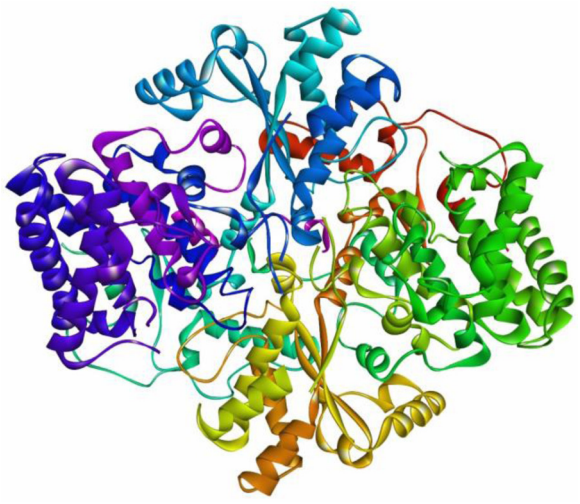


Fig. 3. Structure of MCR receptor.

2.2. Selection of Potent Ligands

2.2.1. Lipinski's Rule of Five

The drug-likeness properties of all 25 ligands were determined using Lipinski's rule of five (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). Molecular weight, logP, number of hydrogen bond acceptors, number of hydrogen bond donors, and molar refractivity of each ligand was determined using this rule [18].

2.2.2. ADME Traits Analyses

Ligands fulfilling the parameters of Lipinski's rule of five were submitted for ADME (absorption, distribution, metabolism, and excretion) potency prediction using Swiss ADME tool of Swiss Institute of Bioinformatics (http://www.swissadme.ch/). The canonical SMILES were retrieved from PubChem and assessed by Swiss ADME tool. Various traits viz. water solubility (Log mol/L), lipophilicity (Log $P_{o/w}$), gastro-intestinal (GI) absorption, blood brain barrier (BBB) permeant, and P-gp substrate were analyzed by this tool. These phytoconstituents were further used for molecular docking mechanism.

2.3. Molecular Docking Analysis

2.3.1. Preparation of Ligands Structures

2D structures of all compounds were retrieved from the PubChem and then subjected to Discovery studio to generate 3D structures and energy minimization [19]. OMEGA 3.0.0 was used to generate conformers of each ligand [20]. OMEGA is known to generate energy minimized molecular structure with their tautomer, ionization state, ring conformation, and stereoisomer to produce broad chemical and structural diversity from a single input structure.

2.3.2. Preparation of Target Protein Structure

The structure of target protein (MCR) was obtained from protein data bank (PDB; ID: 1MRO) [21]. Discovery Studio Client software was implemented to prepare the targeted receptor structure by removing water molecule, heteroatoms, and assigned charges and adds hydrogen and missing residues (if present) (Fig. 3). After preparing the structure of the receptor, active site was defined using co-crystal compounds and centroid on all residues within 10 Å co-crystal compounds.

2.3.3. Docking Analysis

After generating the structures of ligands and receptor, molecular docking was analyzed to determine the binding affinity. The calculation of molecular docking was estimated using FRED v3.2.0 from OpenEye Scientific Software [22]. FRED needs a set of input conformers for each ligand which was created by OMEGA 3.0.0. Default parameter of FRED was used for the docking calculations which produced ten poses for each ligand. Ligands showing chemguass4 score were selected for further analysis. Binding interaction of best-docked poses was observed using Discovery Studio client v16.1.0 [19].

3. Results

3.1. Drug-Likeness Properties of Phytocomponents

The drug-likeness properties of all the selected phytoconstituents of safflower oil were predicted by Lipinski's rule of five. Various parameters viz. molecular weight, LogP, number of hydrogen bond acceptors, number of hydrogen bond donors, and molar refractivity of the selected phytoconstituents are shown in Table 1. According to Lipinski's rule of five, 9 compounds (methyl tetradecanoate, 3-isopropyl-6-methylenecyclohex-1-ene, trans-2,4-decadienal, cis-6-nonenal, limonene, syringic acids, matairesinol, acacetin, and 2,5-octanedione) were identified as the most appropriate ligands satisfying all the criteria.

3.2. Analysis of ADME Properties

The ADME characteristics of 9 selected compounds of safflower oil are illustrated in Table 2. Results revealed the absence of Lipinski's violation and approval of drug-likeness attributes of methyl tetradecanoate, 3-isopropyl-6-methylenecyclohex-1-ene, trans-2,4-decadienal, cis-6-nonenal, limonene, syringic acids, matairesinol, acacetin, and 2,5-octanedione. All the compounds showed hydrophilic and lipophilic properties. However, 2,5-octanedione and methyl tetradecanoate showed maximum water solubility and lipophilicity of -0.62 (Log mol/L) and 3.88 (Log $P_{o/w}$), respectively. Except 3-isopropyl-6-methylenecyclohex-1-ene and limonene, all other compounds showed high GI absorption. On the other hand, syringic acids, matairesinol, and acacetin exhibited no permeation via BBB. No P-gp substrate was observed for all the compounds.

3.3. Molecular Docking Analysis

Table 3 illustrates the binding affinity values of methyl tetradecanoate, 3-isopropyl-6-methylenecyclohex-1-ene, trans-2,4-decadienal, cis-6-nonenal, limonene, syringic acids, matairesinol, acacetin, and 2,5-octanedione with MCR receptor. Results showed maximum binding interaction of acacetin with MCR with the chemguass4 score of -13.35. Other ligands showed binding affinity in the order of matairesinol (-12.43) > methyl tetradecanoate (-9.25) > cis-6-nonenal (-7.88) > syringic acids (-7.73) > limonene (-7.18) > trans-2,4-decadienal (-7.07) > 3-isopropyl-6-methylenecyclohex-1-ene (-7.01) > 2,5-octanedione (-7.0.). Molecular binding images between each ligand and MCR are also shown in Table 3.

4. Discussion

Methanogenesis occurs not only in natural anaerobic environment but also in the digestive tract of animals [23]. Methanogens convert various substrates into CH_4 via methanogenesis in order to obtain energy for their growth and metabolism. Approximately 600 million metric tons of CH_4 are released per year in the ecosystem via methanogenesis process. The global warming impact of CH_4 is considered about 30 folds higher than that of CO_2 which indicates the production of CH_4 a major threat for the environment [24].

Table 1
Phytocomponents of safflower oil analyzed by Lipinski's rule of five.

S. No.	Phytocomponents	Molecular Formula / Mass	logP	Number of Hydrogen Bond Acceptors	Number of Hydrogen Bond Donors	Molar Refractivity
1	Methyl tetradecanoate	$C_{15}H_{30}O_2$ / 242.4	4.41	02	00	82.6
2	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$ / 256.42	4.72	02	00	88.07
3	7-hexadecenoic acid, methyl ester	$C_{17}H_{32}O_2$ / 268.4	4.82	02	00	91.2
4	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$ / 284.5	5.33	02	00	99.0
5	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$ / 326.6	6.25	02	00	115.4
6	7,10-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$ / 294.5	5.23	02	00	99.8
7	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$ / 354.6	6.86	02	00	126.34
8	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$ / 382.7	7.47	02	00	137.27
9	5-hydroxymethylfurfural	$C_6H_6O_3$ / 126.11	0.75	03	01	27.89
10	1-octene	C_8H_{16} / 112.21	2.59	00	00	42.11
11	3-isopropyl-6-methylenecyclohex-1-ene	$C_{10}H_{16}$ / 136.23	2.54	00	00	49.13
12	Trans-2,4-decadienal	$C_{10}H_{16}O$ / 152.23	2.43	01	00	48.48
13	Caryophyllene	$C_{15}H_{24}$ / 204.35	3.96	00	00	75.11
14	Cis-6-nonenal	$C_9H_{16}O$ / 140.22	2.54	01	00	47.18
15	Limonene	$C_{10}H_{16}$ / 136.23	2.53	00	00	49.19
16	Linoleic acid	$C_{18}H_{32}O_2$ / 280.4	4.52	02	01	94.52
17	Palmitic acid	$C_{16}H_{32}O_2$ / 256.42	4.32	02	01	88.26
18	Stearic acids	$C_{18}H_{36}O_2$ / 284.5	4.93	02	01	99.19
19	Syringic acids	$C_9H_{10}O_5$ / 198.17	0.86	05	02	41.8
20	Matairesinol	$C_{20}H_{22}O_6$ / 358.4	3.14	06	02	92.72
21	Acacetin	$C_{16}H_{12}O_5$ / 284.26	1.65	05	02	67.17
22	Acetoin	$C_4H_8O_2$ / 88.11	0.83	02	01	23.26
23	2,5-octanedione	$C_8H_{14}O_2$ / 142.2	2.0	02	00	42.77
24	Caproic acid	$C_6H_{12}O_2$ / 116.16	1.26	02	01	33.59
25	Tetradecene	$C_{14}H_{28}$ / 196.37	1.45	02	00	41.66

Table 2
ADME properties of selected phytocomponents of safflower oil.

S. No.	Phytocomponents	SMILES	Water Solubility (Log mol/L)	Lipophilicity (Log $P_{o/w}$)	GI Absorption	BBB Permeant	P-gp Substrate	Lipinski's Violation	Drug Likeness
1	Methyl tetradecanoate	CCCCCCCCCCCCCC(= O)OC	-4.52 (moderately soluble	3.88	High	Yes	No	00	Yes
2	3-isopropyl-6-methylenecyclohex-1-ene	CC(C1CCC(= C)C = C1)C	-2.79 (soluble)	2.65	Low	Yes	No	00	Yes
3	Trans-2,4-decadienal	CCCCCC = C/C = C/C = O	-2.44 (soluble)	2.67	High	Yes	No	00	Yes
4	Cis-6-nonenal	O = CCCCC/C = C\CC	-1.78 (very soluble)	2.34	High	Yes	No	00	Yes
5	Limonene	CC1 = CCC(CC1)C(= C)C	-3.5 (soluble)	2.72	Low	Yes	No	00	Yes
6	Syringic acids	COc1cc(cc(c1O)OC)C(= O)O	-1.84 (very soluble)	1.54	High	No	No	00	Yes
7	Matairesinol	COc1cc(ccc1O)C[C@H]1C(= O)OC[C@@H]1Cc1ccc(c(c1)OC)O	-4.06 (moderately soluble)	2.47	High	No	No	00	Yes
8	Acacetin	COc1ccc(cc1)c1cc(= O)c2c(o1)cc(cc2O)O	-4.14 (moderately soluble)	2.56	High	No	No	00	Yes
9	2,5-octanedione	CCCC(= O)CCC(= O)C	-0.62 (very soluble)	1.76	High	Yes	No	00	Yes

Methanogens reduce CO_2 into CH_4 in the hind gut of equines [25]. However, the members of Equidae family, particularly horses produce 3–4 times lesser CH_4 than other ruminants [26]. This variation depends on diversified factors, including contrasting microflora in the digestive tract of ruminants and horses as well as difference in the gut anatomy of horses [26]. In spite of the comparatively reduced emission of CH_4 from horses, the strategy to reduce its emission in the ecosystem is required, considering its significant contribution in global warming effect.

Over the past few years, several *in vitro* strategies have been implied to mitigate the emission of CH_4 from livestock [27]. The dietary manipulation is considered as one of the most potential and practiced approaches to reduce the rate of CH_4 emission from horses. As a matter of fact, the supplementation of plant extracts, probiotics, plant metabolites, exogenous enzymes, and organic acids as additives in the diet of animals alter the gut microflora, thereby affecting the fermentation kinetics and leading to the reduced emission of CH_4 [3]. In addition, dietary supplements also improve the quality of the feed and change the proportion of the diet effectively which ultimately affects the metabolism of gut microflora, followed by significant alteration in the fermentation kinetics [27]. However, the supplementation of diversified additives in the pricey feed and analyzing its *in vitro* or *in vivo* CH_4 mitigation characteristics in livestock is a time consuming and expensive process. Thus, it is imperative not only to save the cost of *in vitro* or *in vivo* experiments but also implement short-term screening experimental plan by finding an alternative strategy in order to find suitable anti-methanogenic agents.

The computational tools have been identified as an effectual alternative approach to save time and resources for veterinarians. Molecular docking mechanism of certain ligands with the target receptor has been proved to be an ideal and inexpensive screening technique [12]. As we know that methanogens require MCR for the methanogenesis process, thus, this has emphasized researchers to target MCR via computational techniques as a new strategy towards the mitigation of CH_4 from animals. Previous *in silico*

Table 3
Molecular docking analysis of selected compounds of safflower oil with MCR receptor.

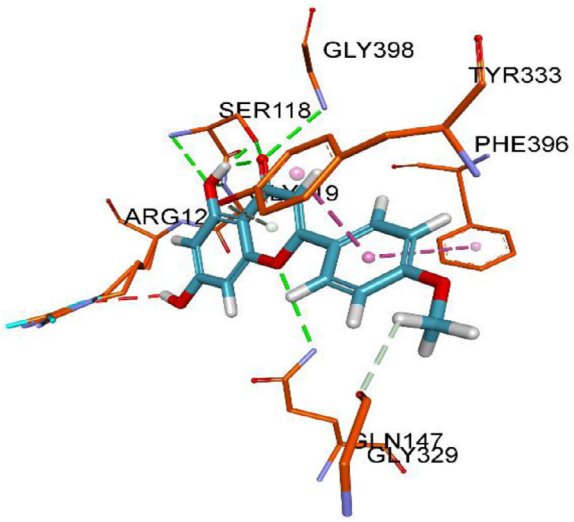
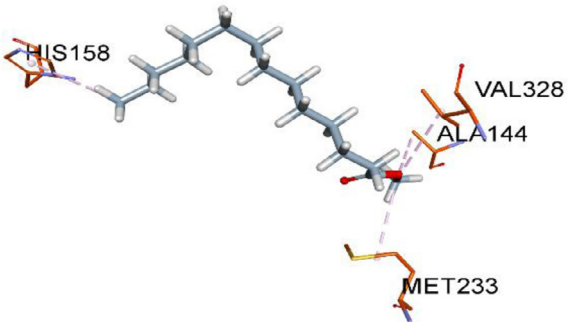
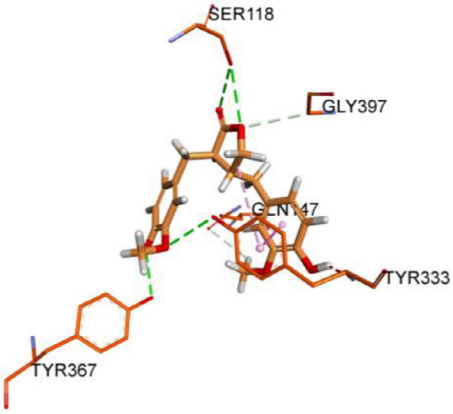
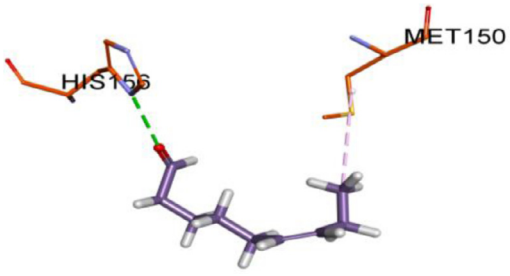
S. No.	Compounds	Chemguass4 Score	Binding Interaction
1	Acacetin	-13.35	
2	Matairesinol	-12.43	
3	Methyl tetradecanoate	-9.25	
4	Cis-6-nonenal	-7.88	

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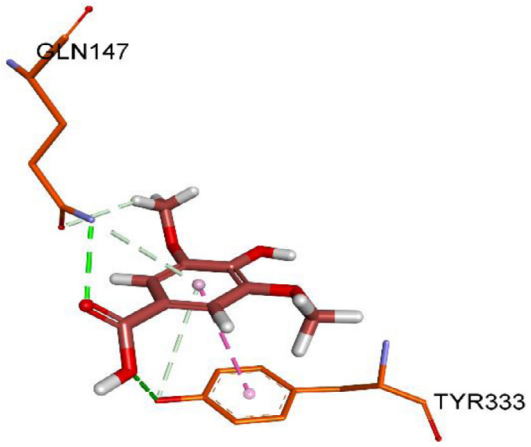
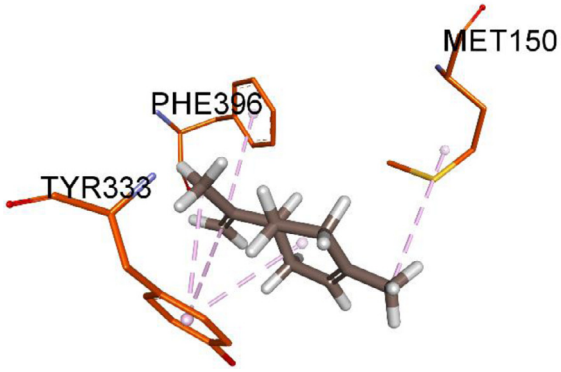
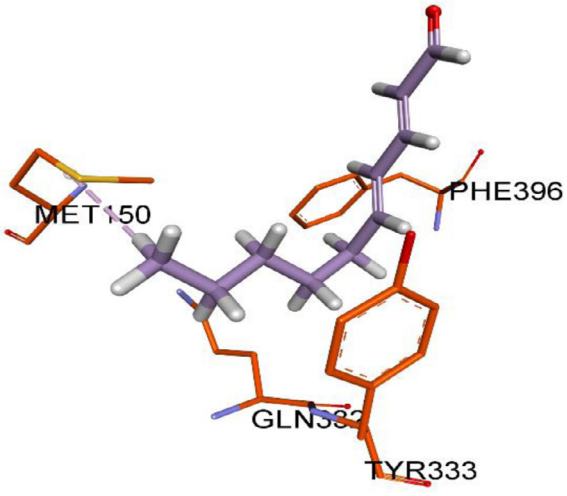
Table 3 (*continued*)

S. No.	Compounds	Chemguass4 Score	Binding Interaction
5	Syringic acids	-7.73	
6	Limonene	-7.18	
7	Trans-2,4-decadienal	-7.07	

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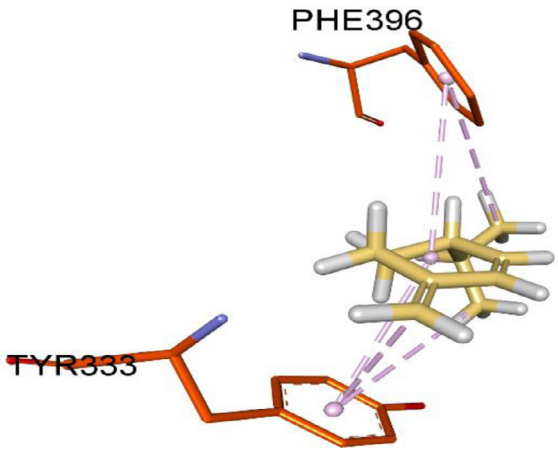
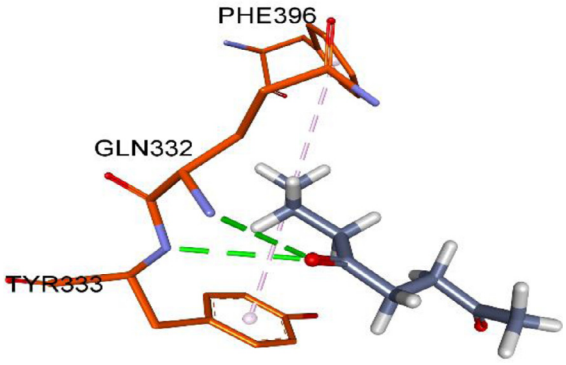
study demonstrated anti-methanogenic attributes of plant metabolites by targeting MCR. Findings reported 9,10-anthracenedione, 1,8-dihydroxy-3-methyl, phthalic acid isobutyl octadecyl ester, and diisooctyl phthalate of *Rheum* sp. as potential anti-methanogenic agents in ruminants via molecular modeling approaches [28]. Likewise, Dinakarkumar et al. [1] studied a total of 168 compounds of 11 different plants towards the mitigation of CH_4 from ruminants by targeting MCR via *in silico* tools. Study reported rosmarinic acid, biotin, α-cadinol, and 2,4,7,9-tetramethyl-5decyn4,7diol as the most effective compounds with MCR inhibitory characteristics. Khusro et al. [9] depicted the pivotal CH_4 mitigation role of certain components, particularly 3,5-bis(1,1-dimethylethyl)-phenol, kaempferol, moringynfghjkne, niazimisin, and tetradecanoic acid of *Moringa oleifera* by analyzing higher binding interaction of these compounds with MCR via Hex 8.0.0 tool.

In the present *in silico* study, acacetin, matairesinol, methyl tetradecanoate, cis-6-nonenal, syringic acids, limonene, trans-2,4-decadienal, 3-isopropyl-6-methylenecyclohex-1-ene, and 2,5-octanedione of safflower oil surpassed all the parameters of Lipinski's rule of five. Generally, according to drug-likeness criteria

Table 3 (*continued*)

S. No.	Compounds	Chemguass4 Score	Binding Interaction
8	3-isopropyl-6-methylenecyclohex-1-ene	-7.01	
9	2,5-octanedione	-7.0	

of suitable ligand, molecular mass should be <500 Da, hydrogen bond donor should be <5, hydrogen bond acceptor should be <10, lipophilicity should be <5 (log p), and molar refractivity should range from 40 to 130 [29]. Likewise, ADME analysis suggested the drug-likeness characteristics of all ligands with no Lipinski's violation. Further, in this context, we evaluated the role of safflower oil-associated all 9 selected compounds as potential inhibitors of MCR which showed maximum binding interaction of acacetin with MCR with the chemguass4 score of -13.35. Other ligands showed comparatively lower binding affinity. This investigation established the first *in silico* report on simulating CH_4 mitigating trait of safflower oil-associated specific bioactive compounds by targeting MCR as receptor. However, our previous *in vitro* study had successfully depicted CH_4 mitigation from horses using safflower oil as an ideal feed supplement [6]. The current *in silico* docking study suggested that the reduced emission of CH_4 from horses after safflower oil supplementation (as discussed in our previous *in vitro* study [6]) might be due to the high binding affinity of safflower oil-associated certain compounds with the MCR, followed by the inhibition of MCR catalytic trait, thereby inhibiting the methanogenesis mechanism.

5. Conclusions

In a nutshell, among 25 selected compounds of safflower oil, 9 compounds satisfied the essential criteria of Lipinski's rule of five. Further, *in silico* assessment exhibited potential binding of those 9 phytocompounds with MCR receptor. Molecular docking simulation showed maximum binding interaction of acacetin with MCR with the chemguass4 score of -13.35. On the other hand, rest of the compounds exhibited comparatively lower binding affinity. Thus, findings of this study indicated greater specificity of acacetin, matairesinol, methyl tetradecanoate, cis-6-nonenal, syringic acids, limonene, trans-2,4-decadienal, 3-isopropyl-6-methylenecyclohex-1-ene, and 2,5-octanedione with MCR binding site and suggested pivotal role of safflower oil-associated these bioactive compounds as ideal anti-methanogenic agents in equine industries.

Acknowledgement

The authors acknowledge Researchers Supporting Project No: (RSP2022R465), King Saud University, Riyadh, Saudi Arabia for funding this research work.

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