

LOCALIZATION AND ORIENTATION OF METHOXY FLAVONOIDS IN DPPC BILAYERS: EFFECT ON THEIR ANTI-PROLIFERATIVE ACTIVITY

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ABSTRACT

Objective: Flavones and flavonols are an important class of naturally occurring flavonoids. They are well known for their pharmacological activity. This activity is associated with the ability of flavones and flavonols to influence membrane-dependent processes. In this paper, we have reported localization, orientation and interaction, of four synthesized flavone/flavonols with 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) bilayers. These are compared with standard flavone; chrysin (CHY) and flavonol Quercetin (QUE).

Methods: The molecules studied are 4'-methoxy flavone (MF), 3',4'-dimethoxyflavone (DMF), 4'-methoxyflavonol (MF-ol) and 3',4'-dimethoxyflavonol (DMF-ol). The techniques used are Differential Scanning Calorimetry (DSC) and multi-nuclear NMR.

Results: Highest binding to lipid bilayers is shown by DMF, followed by QUE. Based on DSC studies it is seen, that maximum interaction of MF and DMF, takes place with the hydrophobic core of lipid bilayers. DMF-ol shows formation of a heterogeneous system at higher concentrations. The ¹H NMR spectra of unilamellar vesicles of DPPC, incorporated with MF, DMF and MF-ol shows significant interaction of these compounds with the alkyl chain of the hydrophobic core. MF, DMF and MF-ol acquire parallel orientation in bilayers with the B-ring pointing towards hydrophobic core, while DMF-ol acquire mixed orientation. This may be ascribed to the presence of two methoxy and one hydroxyl group on the B-ring of DMF-ol which hinders its partitioning inside the hydrophobic core of lipid bilayer. Multi-lamellar vesicles (MLV) of DPPC incorporated with flavones, show maximum increase in Chemical Shift Anisotropy in ³¹P spectrum of DMF. This is followed by MF. DSC.

Conclusion: NMR and binding studies indicate that DMF is partitioned deeply inside the hydrophobic core, while MF, MF-ol and DMF-ol are mostly located in the vicinity of sn-glycero region. Therefore, we conclude that DMF which penetrates deepest inside the hydrophobic core also shows the highest anti-proliferative activity against K562 and MCF-7 cancer cell lines. Its activity is also better than CHY.

Keywords: Flavones, Flavonols, DPPC, DSC, NMR, Chemical shifts, CSA

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INTRODUCTION

Flavonoids are produced ubiquitously in plants. Several of them can be found in the diet or in traditional herbal medicines. Dietary flavonoids and other polyphenols have shown great potential as cancer chemopreventive agents in cell culture studies [1-3]. However, their oral bioavailability is poor due to which, they are hardly used as chemotherapeutic drugs [4, 5]. The extensive conjugative metabolism in the intestine and liver is responsible for their poor *in vivo* activity [6, 7]. The challenge is to modify the flavonoid moiety so as to facilitate the *in vivo* activity and thereby, increase their bioavailability. It has been reported [8] that replacement of hydroxyl group in flavones with methoxy groups reduce the degree of metabolic removal whilst retaining anti-proliferative potency. O-methylation provides polyphenols with protection against hepatic metabolism [9]. Their permeability across cell membranes is also increased [10]. Methoxy flavonoids are found naturally in plants [11, 12]. Due to their increased lifespan in our body, methoxy flavonoids are much more capable to induce potential health effects *in vivo* as compared to their un-methoxylated counterparts [13]. Methoxy substituted flavonols have shown better anti-inflammatory and anticancer activity against human prostate cancer cell lines as compared to their polyphenolic counterpart [14, 15].

The anti-proliferative activity of the methoxy flavonoids have been explored recently [16-18]. However, there are very few studies on their membrane interaction. It is known that the great bioactivity of flavonoids is closely linked to their ability to interact with membranes [19]. It is therefore important to study their localization and type of interaction inside the membranes. We have synthesized some mono- and di-methoxy flavones and flavonols [4'-methoxyflavone (MF), 3', 4'-dimethoxyflavone (DMF), 4'-methoxy-

flavonol (MF-ol), and 3', 4'-dimethoxyflavonol (DMF-ol)] (fig. 1). Their interaction, localization and orientation inside the lipid bilayer of DPPC model membranes have been studied. The interaction behavior, of methoxy flavonoids have been compared with the standard unmethoxylated polyphenols, chrysin (CHY) and quercetin (QUE). The antioxidant and anti-proliferative activity of methoxy flavonoids has also been studied and compared with CHY and QUE. A correlation between the interaction of the flavonoids with the lipid bilayer and their anti-proliferative activity has been established.

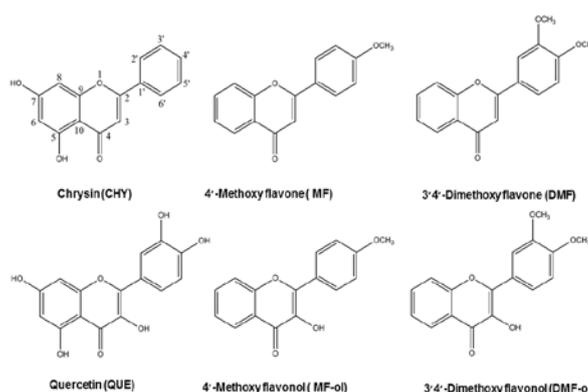


Fig. 1: Structure of Chrysin (CHY), 4'-Methoxyflavone (MF), 3', 4'-Dimethoxyflavone (DMF), Quercetin (QUE), 4'-Methoxyflavonol (MF-ol), and 3', 4'-Dimethoxyflavonol (DMF-ol)

MATERIALS AND METHODS

Materials

Chrysin, quercetin, 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), benzoic acid, phosphorous pentachloride (PCl₅) and 2-hydroxyacetophenone were purchased from Sigma chemicals Co. USA. The chemicals used for synthesis were purchased from SD Fine chemical, India and were of A. R. grade. The solvents used were of A. R. grade.

Synthesis

The synthesis of compound 4'-methoxyflavone (MF) and 3', 4'-dimethoxyflavone (DMF), was carried out according to the Claisen-Schmidt condensation reaction, using Lithium hydroxide (LiOH. H₂O) as a base catalyst. 4'-methoxyflavonol (MF-ol), and 3', 4'-dimethoxyflavonol (DMF-ol) were synthesized from 2-hydroxyacetophenone and 4-substituted benzaldehydes in two steps. (i) Claisen Schmidt reaction resulting in the formation of the chalcone. (ii) Algar-Finland-Oyamanda reaction resulting in the synthesis of the flavonols. A suspension of 4-methoxybenzaldehyde (for MF-ol) or 3, 4-dimethoxybenzaldehyde (for DMF-ol) and 2-hydroxyacetophenone in ethanol was cooled and KOH solution was added dropwise. The reaction mixture was stirred at room temperature. CH₂Cl₂ was added and the organic layer was washed with H₂O, dried over sodium sulphate, and concentrated *in vacuo*. The oily residue was dissolved in dioxane and ethanol and NaOH solution was added. To this H₂O₂ was added dropwise. The reaction mixture was stirred in an ice bath and subsequently at room temperature, resulting in a yellow suspension. After acidification with 2M HCl the precipitate was filtered and dried. The crude product was purified by passing it through silica gel GF column using chloroform as mobile phase, to give pure products. The ¹H and

¹³C chemical shifts of CHY, MF, DMF, QUE, MF-ol and DMF-ol in DMSO-d₆ at 323K are given in table 1.

NMR and DSC experiments

NMR experiments were recorded on a BRUKER AVANCE 500 MHz NMR spectrometer. 2D-COSY and 2D-NOESY spectra were recorded using standard pulse sequences [20, 21], with a mixing time of 400 ms. [31]P and ¹³C experiments were carried out with a relaxation delay of 1s, using broadband ¹H decoupling. NMR software Topspin 2.0 was used for data processing. DSC measurements were performed on differential scanning calorimeter VP-DSC (Microcal, Northampton, MA, USA) using procedure used earlier [22]. Repeated scans for the same samples were generally super imposable. Data were analyzed with the software ORIGIN.

Sample preparation

Multi-lamellar vesicles (MLV's) from DPPC were prepared using standard procedure [23]. The desired quantity of DPPC and drug was dissolved in chloroform. The solvent was evaporated with a stream of nitrogen so as to deposit a lipid film on the walls of the container. The last traces of the solvent were removed under vacuum. MLV's sample thus prepared, was hydrated with the required amount of D₂O at pH 7.2, followed by incubation in water bath at 50 °C with repeated vortexing. The lipid concentration for NMR samples was maintained at 100 mmol. The concentrations of the flavones were varied from 10 to 50 mmol. For DSC experiments, samples were prepared by mixing the lipid and drug solutions to obtain drug/lipid ratios ranging from 1:20 to 1:2 by maintaining the lipid concentration to 50 mmol. Unilamellar vesicles (ULV's) for NMR experiments were prepared by sonicating the lipid dispersions, using a Branson Sonicator-450 at 50% duty cycles till optical clarity was obtained.

Table 1: ¹H and ¹³C NMR chemical shifts (ppm) for flavonoids in DMSO-d₆ at 323K

¹ H ¹³ C	CHY ¹ H ¹³ C	MF ¹ H ¹³ C	DMF ¹ H ¹³ C	QUE ¹ H ¹³ C	MF-ol ¹ H ¹³ C	DMF-ol ¹ H ¹³ C							
H3	C2	6.9(s)	163.7	6.9(s)	162.8	7.0(s)	163.3	6.2(s)	147.4	8.1(d)	146.1	8.1(d)	146.1
H5	C3	6.2(s)	105.7	8.0(d)	106.0	8.1(d)	106.3	6.4(s)	136.2	7.5(t)	133.9	7.5(t)	138.9
H6	C4	6.5(s)	182.3	7.8(t)	177.5	7.5(t)	177.5	7.7(s)	176.4	7.8(t)	173.2	7.8(b)	177.3
H7	C5	8.1(d)	162.0	7.5(t)	123.9	7.8(t)	124.0	6.9(d)	161.2	7.7(d)	124.9	7.8(b)	124.0
H8	C6	7.6(d)	99.6	7.8(d)	125.8	7.8(d)	125.8	7.6(d)	98.7	8.2(s)	125.1	7.8(s)	125.3
H2'	C7	7.6(m)	164.9	8.1(d)	125.3	7.6(d)	125.2	9.2(s)	164.4	7.1 (s)	125.1	7.9(d)	125.0
H3'	C8	7.6(m)	94.6	7.1(d)	118.9	7.2(d)	123.9	12.5(s)	93.8	7.2(s)	118.6	7.2(d)	119.0
H4'	C9	8.6(d)	158.0	7.1(d)	156.2	7.7(d)	156.2	10.7(s)	157.0	8.2(s)	154.9	3.9(s)	155.0
H5'	C10	12.7(s)	104.5	8.1(d)	134.5	3.9(d)	134.5	9.2(s)	98.7	3.87(s)	138.5	9.5(s)	134.0
H6'	C1'	10.7(s)	131.3	3.9(s)	131.5		119.0	9.2(s)	122.5	9.26(s)	121.8		121.9
4'-OCH ₃	C2'		126.9		128.7		110.3		115.6		129.8		112.1
3-OH	C3'		129.6		115.1		152.6		148.2		114.5		149.2
5-OH	C4'		132.4		163.3		149.7		145.5		160.9		151.1
7-OH	C5'		129.6		115.1		112.5		116.1		114.5		112.4
3'-OH	C6'		126.9		128.7		120.5		115.7		129.8		122.1
4'-OH	CH ₃				56.1		56.3,56.5				56.0		56.2,56.3

Determination of drug-MLV binding constants

Drug-MLV binding constants were determined by centrifugation method [24]. MLV's were prepared by varying lipid concentration from 0.25 mg/ml to 2 mg/ml with fixed drug concentration of 100 μM. This corresponds to a drug: lipid ratios in the range 1:2.5 to 1:20. The resulting solutions were incubated and subsequently transferred into ultracentrifuge tubes. Separation of liposomes from the aqueous phase was achieved by centrifugation. Optical density of 100μM solution of the pure drug was measured at a wavelength range of 220-400 λ. The amount of drug bound to liposomes was determined from the difference in optical density measured for the pure drug and that of the supernatant. The drug-liposome apparent binding constant (k) has been analyzed using the double reciprocal plot of fraction bound versus lipid concentration which yields a straight line with a slope 1/k.

Determination of antioxidant activity by DPPH assay

Varying concentrations of each drug (0-200 μg/ml, 0.5 ml in methanol) was mixed with methanolic solution of DPPH (0.1 mmol,

1.0 ml) and Tris-HCl buffer (0.1 M, pH 5.5, 1.0 ml) to make a total volume 2.5 ml. The absorbance of the sample was measured at 517 nm, after 0.5 h of incubation [25]. The reaction solution without DPPH was used as a blank test. Measurements were performed in triplicate. Free radical scavenging activity of the drug was measured as the difference in absorbance between the test sample and the control (i.e. sample without drug). Concentration required for a 50% reduction (IC₅₀ in μg/ml) of DPPH radical solution was determined graphically.

Determination of anti-proliferative activity

Anti-proliferative activity was evaluated by the Sulforhodamine B assay method [26]. Two different cell lines, K562 and MCF-7 were used. Cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mmol of L-glutamine. The test compounds were dissolved in dimethyl sulfoxide and diluted suitably before adding them to the culture medium. After incubation at standard conditions for 48 h, percent growth inhibition of cells has been calculated.

RESULTS AND DISCUSSION

Binding studies

Fig. 2 shows a plot of fraction of drugs bound to MLV's with increasing concentration of lipid. In all cases, an increase in binding affinity is observed, with increasing concentration of lipid. 75–78% binding is shown by QUE, DMF and MF, CHY shows nearly 70% binding, while MF-ol and DMF-ol show only 62–65% binding at the highest concentration. Double inverse plot of fraction of drug bound vs inverse of lipid concentration (inset fig. 2) has been used to calculate the binding constants. The results indicate, that these molecules bind to the MLV's with a variable degree of affinity and follow the order QUE>DMF>MF>CHY>MF-ol>DMF-ol. The apparent binding constants measured are: QUE: 1073 M⁻¹, DMF: 690 M⁻¹, MF: 559 M⁻¹, CHY: 472 M⁻¹, MF-ol: 390 M⁻¹ and DMF-ol: 293M⁻¹. It is noted that QUE, which has two hydroxyl groups on the B-ring, shows maximum binding. MF and DMF, which have one and two methoxy groups respectively on the B-ring show considerable binding. MF-ol and DMF-ol show least binding. It appears that the penetration of the B-ring inside the lipid bilayer is responsible for the binding of flavonoids. The higher binding of flavones (MF and DMF) compared to that of flavonols (MF-ol and DMF-ol), may be due to the absence of-OH group on the B-ring. Possibly, presence of-OH group in addition to methoxy group on the B-ring, hinders its penetration inside the hydrophobic core. The methoxy groups present in B-ring of flavones may facilitate penetration. The binding results therefore suggest that the number and type of groups present on the B-ring of flavonoids play a significant role in their penetration inside the DPPC lipid bilayer.

DSC studies

DSC is a sensitive technique for studying the effect of drugs on the packing order of the lipid bilayers. Thermotropic aspect of drug-lipid interactions can be studied by examining changes in the melting point and the shape of the DSC thermograms [27]. Fig. 3 shows DSC thermograms of lipid bilayers incorporated with increasing concentrations of CHY, MF, DMF, QUE, MF-ol and DMF-ol. In each case, the lower most graphs (a) represent the thermogram for lipid bilayers alone. Here, the pre-transition temperature (T_p) at 34.1 °C indicates the mobility of the choline part of the polar head of DPPC. Mobility of the alkyl chain is reflected in the main transition (T_m) at 41.9 °C. In the case

of flavonols QUE and MF-ol the pre-transition peak is abolished. Abolition of such a peak, indicates a strong interaction of these molecules, with the polar head-group region of the lipid bilayer. However, pretransition peak is present in thermogram of MF, DMF and DMF-ol. The presence of such a peak indicates the interaction of these molecules with head group region of lipid bilayer [28]. In the case of MF and DMF the value of pretransition peak decreases with increase in concentration of drug, but in CHY and DMF-ol it initially decreases and at concentrations higher than 1:10 (drug: lipid molar ratio) it increases.

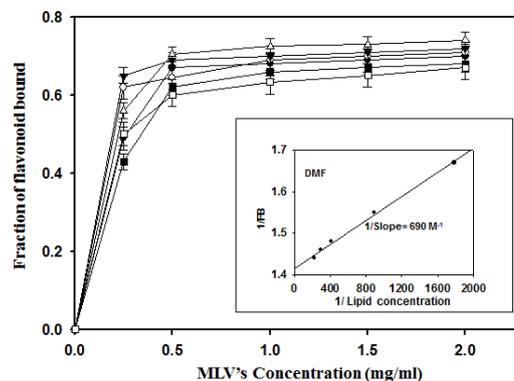


Fig. 2: Binding of CHY (●), MF (○), DMF (▼), QUE (△), MF-ol (■) and DMF-ol (□), with DPPCMLV's. The fraction of drug bound (FB) has been determined by the centrifugation method, as described in the text. The inset fig. shows the double reciprocal plot for DMF

A decrease in the main transition temperature on addition of the compounds indicates an interaction of the molecules with the hydrophobic core of the lipid bilayer. The maximum decrease in T_m value is observed for DMF (at drug/lipid molar ratio of 1:2) i.e. 3.7 °C as compared to pure DPPC bilayer (table 2), indicating a strong interaction with the hydrophobic core which enhances the fluidity of the lipid bilayers.

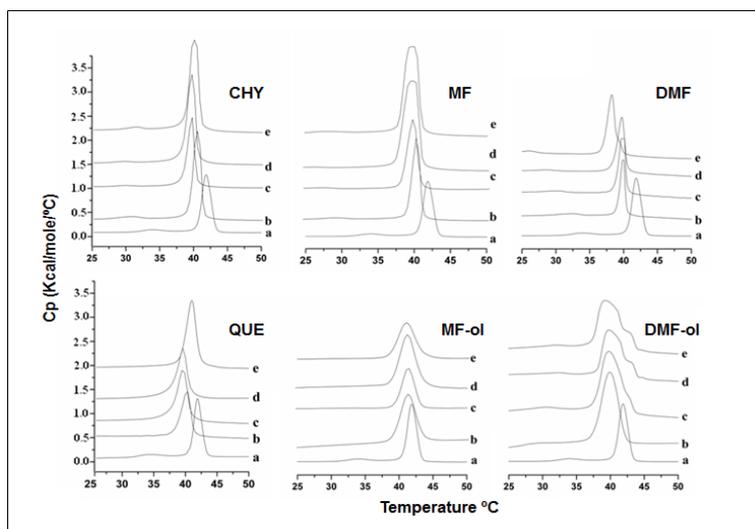


Fig. 3: DSC heating curves of hydrated MLV's of DPPC (50 mmol) containing drugs, at drug/lipid molar fractions; a=0.0; b=0.05; c=0.1; d=0.2; e=0.5

Table 2: Pretransition (T_p) and main transition (T_m) temperatures of DPPC (50 mmol) with varying drug/lipid molar ratios

Drug/DPPC	CHY T _p T _m	MF T _p T _m	DMF T _p T _m	QUE T _p T _m	MF-ol T _p T _m	DMF-ol T _p T _m T _{m1}
Pure DPPC	34.1 41.9	34.1 41.9	34.1 41.9	34.1 41.9	34.1 41.9	34.1 41.9--
1:20	31.0 40.6	29.3 40.3	32.5 40.0	-- 40.2	-- 41.5	29.6 39.9--
1:10	-- 39.8	28.3 39.8	30.6 39.9	-- 39.7	-- 41.4	30.5 39.8 41.7
1:5	30.5 39.9	28.0 39.7	28.9 39.7	-- 39.6	-- 41.2	32.1 39.7 41.9
1:2	31.6 40.1	-- 39.7	26.3 38.2	-- 39.7	-- 41.1	32.5 39.2 42.8

This is followed by MF with maximum decrease of 2.2 °C. A broadening of T_m peak in the case of MF indicates a decrease in cooperativity of the alkyl chain again indicating its localization, in the hydrophobic core.

In case of CHY and QUE the T_m value decreases up to a concentration of 1:5 (CHY/QUE: lipid) molar ratio after which it increases. This can be explained by the tendency of QUE and CHY to form aggregates when in excess of their solubility in lipid bilayers [29].

The thermogram of MF-ol shows a decrease in T_m with increase in concentration however, the decrease in value is less than that of MF and DMF. This indicates interaction with the hydrophobic core of lipid bilayer. DMF-ol shows a broadening of the main transition peak and appearance of an additional shoulder peak. This new transition peak is observed at a temperature higher than the main transition peak. As concentration of DMF-ol is increased from 1:10 (drug: lipid) to 1:2 (drug: lipid) molar ratio the new peak becomes more prominent. This behavior indicates domain formation and phase separation at higher concentrations of DMF-ol. High concentration of DMF-ol probably induces a heterogeneous system [30]. This means that a homogeneous distribution of the compound within the DPPC bilayers did not occur but rich and poor compound regions are present. It also explains the increase in value of pre-transition peak of DMF-ol with increase in drug concentration.

The above DSC results indicate maximum interaction of MF and DMF with the hydrophobic core. DMF-ol shows interaction with the head group as well as with the alkyl chain of the lipid bilayer at lower drug concentration, but finally forms a heterogeneous system at higher concentrations.

NMR experiments

^1H and ^{13}C NMR spectra of CHY, MF, DMF, QUE, MF-ol and DMF-ol in DMSO-d_6 have been assigned using 2D COSY spectrum, literature data and splitting patterns (table 1). Fig. 4 shows aromatic region of ^1H NMR spectra of drugs alone (spectra a, b, c, d, e, and f) and of drugs incorporated with ULV's of DPPC (spectra a', b', c', d', e' and f'). It is observed that in all cases, the drug signals arising from

aromatic protons become broad on incorporation into lipid bilayers. There are significant amount of chemical shift changes both downfield and up-field in all cases. This is because the molecule is likely to interact by spanning from head to tail region of lipid bilayers involving interaction with both polar as well as hydrophobic chain region. Aromatic protons of MF, DMF and MF-ol show both up-field and downfield shifts. 8H and 6H protons of MF show significant up-field and downfield shifts respectively, indicating strong interactions at these positions with lipid bilayer. DMF and MF-ol when incorporated in DPPC bilayers show significant downfield chemical shift of 5H proton indicating strong interactions at this position. The aromatic region of DMF-ol show distinct behavior than that of MF, DMF and MF-ol on interaction with lipid molecules. In ^1H NMR of ULV of DPPC incorporated with DMF-ol, the peaks of the aromatic region are broadened significantly and additional peaks are observed. This may be due to formation of two different domains as we have discussed in our DSC results. Based on the peak intensity as well as their ^1H - ^1H NOESY spectrum we have characterized the peaks of DMF-ol. Here again, we observe a significant downfield shift of 5H proton indicating strong interaction at this position. CHY and QUE show only downfield shift of the protons as these molecules are likely to bind at the lipid water interface due to presence of hydroxyl groups on the ring A. Presence of hydroxyl group on ring-A mostly shifts the location of the ring to lipid water interface [31, 32].

A comparison of the ^1H NMR spectra of pure DPPC and ULV's of DPPC incorporated with drugs indicates shift of DPPC protons on binding with flavonoids under study (fig. 5). These shifts (so called induced chemical shifts), can be used for predicting the localization of the compounds in the lipid bilayer. Upon binding with CHY and QUE there is significant shift in all the regions of lipid but the largest shift is in the *sn*-glycerol region of the lipid water interface. This indicates their localization in this region. Uniform up-field shift is induced in the DPPC protons on incorporation with MF, DMF and MF-ol (fig. 5). However, interaction of MF and DMF is in the alkyl chain region as well as with the *sn*-glycerol region whereas, that of MF-ol is only in the *sn*-glycerol region. In case of MF-ol and DMF-ol the interaction observed with alkyl and *sn*-glycerol region is comparatively less.

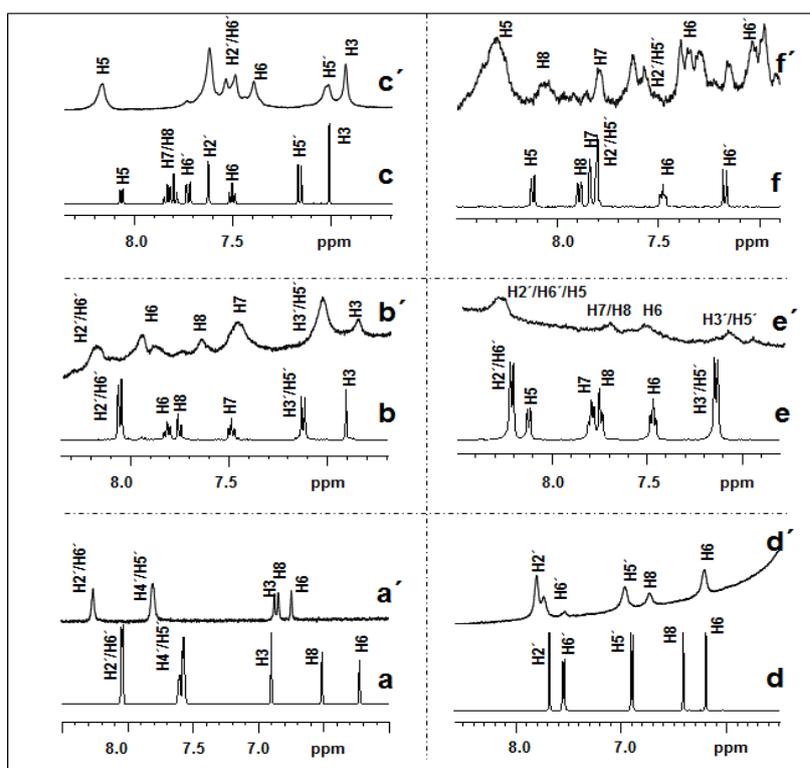


Fig. 4: 500.13 MHz ^1H NMR spectra of aromatic region of pure CHY (a), MF (b), DMF (c), QUE (d), MF-ol (e) and DMF-ol (f) and that of ULV's of DPPC incorporated with CHY (a'), MF (b'), DMF (c'), QUE (d'), MF-ol (e') and DMF-ol (f') in 1:5 drug: lipid molar ratio at 323K in D_2O

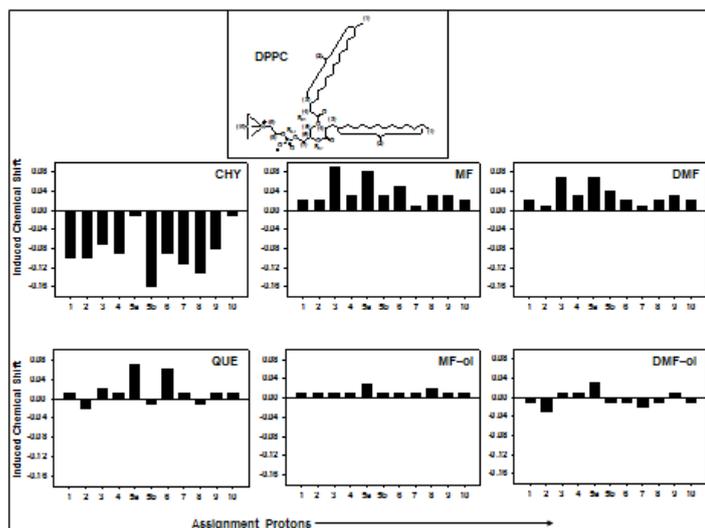


Fig. 5: Ring current induced chemical shift changes in the signals of ULV's of DPPC incorporated with drugs in 1:5 drug/lipid molar ratio at 323K in the presence of CHY, MF, DMF, QUE, MF-ol and DMF-ol. Chemical shifts are either up-field (+) or downfield (-), depending on the orientation of the drug molecule with respect to the bilayer normal

The induced chemical shifts of the lipid protons also indicate the orientation of the drug molecules with respect to the lipid bilayer normal [33]. Drug molecules impart ring current effects on the lipid protons, due to which the lipid resonances are known to shift up-field or downfield. Upon interaction with ring edge the resonances are shifted downfield and on interaction with ring centre it is shifted up-field [34]. Thus, a uniform downfield shift indicates the ring plane to be perpendicular whereas a uniform up-field shift indicates a parallel orientation of the ring plane with reference to the bilayer normal. A mixed orientation of the rings is indicated by the absence of a uniform up-field or downfield shift. A perpendicular orientation is seen in case of CHY with respect to the lipid bilayer normal. MF, DMF and MF-ol show a parallel orientation. QUE and DMF-ol show mixed orientation with respect to bilayer normal.

To probe deeper into the nature of interaction, ^1H - ^1H NOESY spectra of ULV's of DPPC and of those incorporated with drugs have been used. In the spectra of DMF incorporated with ULV's, intermolecular NOE of the methoxy protons with the $-(\text{CH}_2)_n$ protons of lipid bilayer is observed (fig. not shown). This indicates interaction of the methoxy group protons with the hydrophobic core of DPPC bilayer. Though the NOESY spectrum of DMF-ol was noisy, but we were able to locate the intermolecular NOEs of H2', H6' with the head region- $\text{N}(\text{CH}_3)_3$ protons. This indicates that DMF-ol molecule

largely resides in the polar head region with some penetration in the hydrophobic core.

In the ^{13}C NMR of the DPPC ULV's and that of ULV's incorporated with CHY, MF, DMF, QUE, MF-ol and DMF-ol, all the signals arising from drugs are broadened compared to their pure form (fig. 6). The broadening of the signals arises due to an exchange at an intermediate NMR time scale between the bound and free form of these molecules [35]. On the other hand, signals arising from lipid bilayers mostly do not broaden. However, broadening of lipid peaks are observed in the spectrum of ULV vesicles incorporated with MF, DMF and DMF-ol in the region belonging to $-(\text{CH}_2)_n-$, $-(\text{CH}_2)_\beta$ and $-(\text{CH}_2)_\alpha$ groups of the alkyl chain of the lipid showing its interaction with the drug molecule (fig. 6). In ULV vesicles incorporated with other compounds, these peaks remain sharp. This supports our observation that MF, DMF and DMF-ol interact with the alkyl chain of the hydrophobic core. In the case of DMF, QUE and DMF-ol the C5 carbon of the *sn-glycero* region (indicated by * in fig. 6) shows significant up-field shift in its position, thereby indicating interaction of the drug molecule at this position. The ^{13}C spectrum of DMF-ol shows several peaks in the aromatic region in spite of broadening, indicating mobility of these atoms. In other words, the aromatic protons present on these carbon atoms of DMF-ol is not involved in interaction with the lipid protons and thus these carbon atoms give rise to sharp carbon peaks in their ^{13}C NMR spectrum.

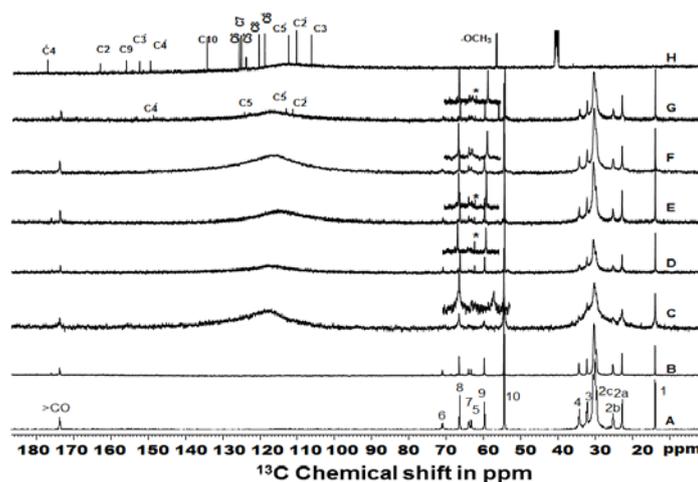


Fig. 6: 125.7 MHz ^{13}C NMR spectra of DPPC ULV's (A) and ULV's of DPPC incorporated with CHY (B), MF (C), DMF (D), QUE (E), MF-ol (F) and DMF-ol (G) in 1:5 drug/lipid molar ratio at 323K in D_2O . The ^{13}C NMR spectrum of pure DMF-ol (H), in DMSO-d_6 is also shown. Peaks for carbon atoms 5 and 7 have been vertically expanded to show upfield shift as indicated by*

^{31}P NMR is sensitive to local motions and the orientation of the phosphate group in the membrane. It has been used for monitoring structural changes and detecting polymorphism in model membranes [36]. Effect of CHY, MF, DMF, QUE, MF-ol and DMF-ol on ^{31}P line shape has been monitored at varying temperature and concentrations. It may be noted here that these molecules do not alter the characteristic line shapes exhibiting

bilayer features of the MLV's of lipid at all concentrations (Supplementary Fig.1). Lipid bilayers give a characteristic broad spectrum with a high field peak and a low field shoulder. Chemical shift anisotropy (CSA) can be measured from the low and high field shoulders of the spectrum (σ_{\parallel} and σ_{\perp} components). The CSA of the phosphate group is used to determine molecular motions near the bilayer head groups [37].

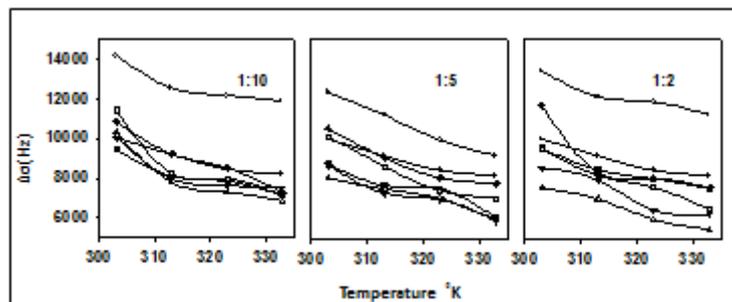


Fig. 7: Change in CSA (Hz) represented by $\Delta\sigma = \sigma_{\perp} - \sigma_{\parallel}$ with temperature at 1:10, 1:5 and 1:2 drug: lipid molar ratios. DPPC MLVs (●), CHY (○), MF (▼), DMF (▲), QUE (■), MFol (□) and DMF (◆)

In case of CHY, QUE and DMF-ol the CSA values decrease upto 1:5 (drug: lipid) molar ratios and then increase at 1:2 (drug: lipid) molar ratio. This may be due to formation of aggregates at higher concentration of drug [29]. This fact is supported by our DSC results.

Thus, based on binding, NMR and DSC studies we can assume that the A-ring of flavonols and flavones are probably located at the lipid/water interface whereas B-ring penetrates into the hydrophobic core of lipid bilayer. MF and DMF is located deep in the hydrophobic core followed by MF-ol. The binding as well as interaction of DMF-ol is hindered by its formation of a heterogeneous system at higher concentrations on incorporation

inside the lipid vesicles. Further, DMF-ol shows a mixed orientation with respect to lipid bilayer normal like QUE.

Anti-oxidant and anti-proliferative activity

DPPH assay has been used to estimate the radical scavenging and antioxidant activity of the flavonols [39]. The radical scavenging activity is known to depend on the number and the position of the phenolic-OH groups [40]. CHY, MF, DMF, QUE, MF-ol and DMF-ol possess OH groups and show highest antioxidant activity. The anti-oxidant property of these molecules follows the order: QUE > CHY > MF-ol > DMF-ol > MF > DMF (table 3), depending on the presence of hydroxyl group. The anti-proliferative activity of the flavonols is shown against K562 and MCF7 cell lines in table 3.

Table 3: *In-vitro* antioxidant and anti-proliferative activity of flavonoids

Flavonoids	DPPH radical scavenging activity IC_{50} ($\mu\text{g}/\text{ml}$) ^a	Percent growth compared to control against different cell lines ^b	
		K562	MCF-7
CHY	20.40	25.9	38.3
MF	46.37	25.4	66.8
DMF	50.20	13.9	14.4
QUE	9.14	12.4	8.3
MF-ol	34.20	32.6	24.9
DMF-ol	42.10	51.2	42.9
ADR	-	-53.4	14.9

^aThe molar drug concentrations required to cause 50% inhibition (IC_{50}) were determined from, dose-response curves. Results represent means \pm SE of at least three different experiments, ^bconcentration of drugs in each case is 10^{-4} $\mu\text{g}/\text{ml}$.

Though QUE interacts significantly only with the sn-glycero region of DPPC, it shows highest anti-proliferative activity against both the cell lines. DMF shows better activity as compared to rest of the compounds. MF shows better activity than MF-ol and DMF-ol against K562. However higher activity is shown by MF-ol as compared to MF against MCF-7 cell line. Therefore, after studying the activity of all the synthesized compounds we can say that, DMF which shows maximum interaction with the hydrophobic core of lipid bilayer also shows better activity as compared to other synthesized compounds, including the standard compound chrysin.

CONCLUSION

The objective of the present study was to establish the effect of substitution at 4'-as well as 3' and 4'-position of the flavones and flavonols on their pharmacological activity. It is proved that localization and interaction of these molecules plays an important

role in their activity. DMF has a strong binding with the lipid hydrophobic core and imparts maximum activity. MF and MF-ol which binds mostly at the lipid/water interface are less active. DMF-ol forms a heterogeneous system on incorporation inside lipid bilayers, which may hinder better interaction with lipid bilayers. It is less active against cancer cell lines K562 and MCF-7. We can therefore, conclude that substitution of a methoxyl group at 4'- and/or 3'-position on the B-ring of the flavone moiety facilitates its partitioning into the hydrophobic core to some extent. This may enhance its anti-proliferative activity against selective cancer cell lines as well. This is also supported by literature reports by Walle *et al.* [41], where they have shown that 5, 7-dimethoxy flavone shows better anti-proliferative activity as well as *in-vivo* bioavailability as compared to their unmethoxylated counterpart. Substitution of a methoxyl group at 4'-position on the B-ring of flavonols, also enhances its activity as well as partitioning into the hydrophobic core of lipid bilayer. Substitution of two methoxyl groups on the B-

ring of flavonol may create steric hinderance in its partitioning inside the lipid bilayer. It is concluded that the substitution of methoxy groups in flavones and flavonols increases its potential as a bioactive compound. The position and number of methoxy group, also play an important role. However, more flavonoid molecules need to be evaluated and other cancer cell lines should be used in order to draw definite conclusions about these structural requirements in regard to methoxylation and antiproliferative activity. These findings can be useful to direct synthesis of new flavonoid-methoxy analogs towards prevention and/or treatment of cancer.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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