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# Structure-biological Activity Relationship of Analogues of 2-Chlorobenzylidenemalononitrile–A Riot-control Agent

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**ABSTRACT**

The riot-control agent 2-chlorobenzylidenemalononitrile (CS) and its ortho-and para-substituted benzylidenemalononitrile (BMN) analogues were synthesised and characterised by spectroscopic techniques (IR, NMR, and mass spectrometry) and microanalysis, and their structure-biological activity relationship studies were carried out to know the factors responsible for sensory irritation. Hydrophobicity of substituted BMNs were determined by high-performance liquid chromatography (HPLC), which is an important determinant of the irritancy. The vapour pressure of a compound is a physico-chemical property important for the assessment of its fate in the environment. The vapour pressures of BMNs were determined by static method by an isoteniscope. A systematic investigation of sensory irritation was carried out by evaluating decrease in respiratory rate ($RD_{50}$) in mice. The $RD_{50}$ as biological parameter was correlated with physical parameters such as hydrophobicity, vapour pressure, size of molecules, and chemical reactivity of its BMNs with dimethylaminoethyl mercaptans (DEAEMs) as a simulant of *protein-SH* group. A biosignifkant correlation (0.75-0.80) was obtained by correlating all the above parameters using multiple linear regression equation.

**Keywords:** Benzylidenemalonitriles, BMNs, riot-control agent, vapour pressure, $RD_{50}$, hydrophobic& high-performance liquid chromatography, diethylaminoethyl mercaptans, 2-chlorobenzylidenemalononitrile, CS, sensory irritants

## 1. INTRODUCTION

Benzylidenemalononitriles (BMNs) are the main products of the condensation of substituted benzaldehydes with malonitrile[1,2]. Derivatives of BMNs have important applications in various areas of chemistry. The BMNs derivatives which incorporate bis-[2-chloroethyl]amino groups, a (bis-chloromethyl) pyrrolidine substituent, or phenylazopyrimidine residue, have been used for chemotherapeutic treatment of cancer[3]. Other substituted BMN compounds are used as pesticides, fungicides, and insecticides[4]. The interest shown in this group of compounds was largely due to the use of 2-chlorobenzylidene-malononitrile (CS) as a riot-control agent'. CS causes irritation of eyes, nose, and respiratory tract with the consequent production of profuse tears and mucus. It is, therefore, also termed as an irritant or tear gas compound'.

In biologically active compounds generally the hydrophobicity[6] plays an important role and is an important determinant of sensory irritancy, hence a suitable measure of their potential use as a riot-control agent is necessary.

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Hydrophobicity of an organic compound has direct bearing on many pharmacological properties. In the case of the irritants, hydrophobicity may be responsible for absorption through the skin of the individual, and therefore, higher hydrophobicity of a chemical may reduce its bioavailability. On the other hand, optimum lipophilicity may be essential for the chemical to reach the appropriate receptor in the human beings to elicit irritancy. Since both the effects are not synergistic, a balance has to be struck in the extent of hydrophobicity for compounds to be effective.

The sensory irritation is an important parameter in relation to occupational exposure. Few reports on the mechanism of irritancy produced by this class of compounds have been reported in the literature'. Some polarographic data and rate of reactions with nucleophile have also been reported in the literature[8-10].

Diethylaminoethyl mercaptan has been used as a simulant of protein-*SH* group and its activity with the BMNs has been studied. Sensory irritation occurs due to the activation of different receptive sites. These sites may be on a single receptor protein or different independent receptors. It was thought that the -*SH* groups of proteins combine with the BMNs at the receptor site to cause sensory irritation. The vapour pressure of a compound is the physico-chemical property important for the assessment of its fate in the environment. The ideal irritant should not have very high vapour pressure at room temperature. The irritant compounds, to be used as riot-control agents, should be volatile at room temperature.

In the present study, sensory irritation of various substituted BMNs has been evaluated by the decrease in respiratory rate (RD,) in mice. An attempt has been made to correlate the $RD_{50}$ as a biological parameter with the other physical parameters like hydrophobicity, vapour pressure, size of molecules, and chemical reactivity of the BMNs with diethylaminoethylmercaptan (DEAEM) to find out the factors responsible for sensory irritation.

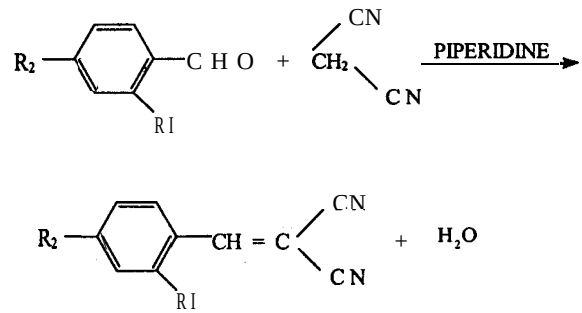
## 2. EXPERIMENTAL PROCEDURE

The reverse-phase high-performance liquid chromatography (HPLC) was performed at room temperature on a Shimadzu ODS LC-6A chromatograph with Shimadzu ODS (5 cm x 4 nm) column. The elution profile was monitored at 254 nm with a UV-visible detector.

The rate constants of BMNs were determined on a Unicam spectrophotometer at 270 nm in dioxane at 25 °C. Melting and boiling points were uncorrected. Elemental analysis (carbon, hydrogen, and nitrogen) was performed using a Heraeus Carlo Erba-1106 elemental analyser. The IR spectrophotometer on a Perkins-Elmer model577 and NMR spectra on ARX-400 instrument were used to confirm the assigned structures.

### 2.1 Synthesis of Substituted BMNs

Various substituted BMNs were synthesised" by the condensation of substituted benzaldehydes with malononitrile in cyclohexane in the presence of piperidine as the base (Fig. 1). The BMNs were purified by recrystallisation and the purity of each compound was found from 98-99 per cent by GLC analysis on column DB-1 using temperature programming 40 °C to 200 °C, carrier gas $N_2$. All the BMNs were characterised by elemental analysis, IR, NMR, and mass spectra. All the substituted BMNs were characterised using IR (cm-$^1$, $\nu_{CN}$=22 1 0-2269, $\nu_{C=C}$ 1580 and 1460) and NMR δ 7.9 to δ 9.0, (S, 1*H*, =*CH*); δ 6.6-8.2 (m, *Ar*, 5*H*). Melting points and microanalysis of all the BMNs are given in Table 1.

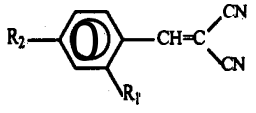



R, = *H, F, Cl, Br, NO,, CH,, OCH,, OH*

$R_2$ = *H, F, Cl, Br, NO,, CH,, OCH,, OH*

**Figure 1. Synthesis of BMNs**

**Table 1. Elemental analysis of BMNs**



| | | Elemental analysis (observed and calculated) (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | C | | H | | N | |
| $R_1$ | $R_2$ | Observed | Calculated | Observed | Calculated | Observed | Calculated |
| H | H | 70.52 | 70.58 | 3.88 | 3.89 | 17.98 | 18.18 |
| Cl | H | 58.59 | 58.90 | 2.62 | 2.65 | 14.87 | 14.82 |
| F | H | 69.70 | 69.70 | 2.72 | 2.90 | 15.98 | 16.27 |
| Br | H | 48.29 | 48.38 | 2.10 | 2.15 | 11.91 | 12.06 |
| $NO_2$ | H | 58.60 | 55.81 | 2.51 | 2.51 | 21.29 | 21.10 |
| $OCH_3$ | H | 70.98 | 71.73 | 4.28 | 4.34 | 15.05 | 15.21 |
| $CH_3$ | H | 77.91 | 78.57 | 4.62 | 4.76 | 16.21 | 16.66 |
| OH | H | 70.12 | 70.58 | 3.40 | 3.52 | 15.97 | 16.40 |
| H | Cl | 62.79 | 63.82 | 2.51 | 2.65 | 14.74 | 14.82 |
| H | Br | 50.96 | 50.86 | 2.05 | 2.15 | 11.96 | 12.06 |
| H | $NO_2$ | 60.30 | 60.30 | 2.15 | 2.10 | 12.47 | 12.50 |
| Cl | Cl | 54.26 | 53.80 | 1.70 | 1.79 | 13.0 | 12.50 |
| H | $OCH_3$ | 70.98 | 71.73 | 4.21 | 4.34 | 15.05 | 15.21 |
| H | $CH_3$ | 78.12 | 78.57 | 4.53 | 4.76 | 16.12 | 16.22 |
| H | OH | 70.12 | 70.50 | 3.40 | 3.52 | 15.97 | 16.40 |
| H | $N(CH_3)_2$ | 72.82 | 73.09 | 5.21 | 5.58 | 20.87 | 21.31 |
| COOH | H | 45.18 | 45.83 | 3.29 | 3.51 | 13.94 | 14.07 |

## 2.2 Determination of Saturated Vapour Pressure Values

The vapour pressure[12] determinations were carried out by static method in an isoteniscope[9]. Prior to vapour pressure measurements, each substituted malononitrile was degassed by a freezing and thawing process. The temperature was maintained constant in a thermostatic water bath to an accuracy of ± 0.025 °C.The pressure was measured from the difference in heights of the mercury columns of the isoteniscope by a cathetometer (OSAW, India) corrected to 0.00 1 mm *Hg*. Most of the compounds did not have sufficient volatility which could be measured at room temperature. Hence, the vapour pressures were determined at three or more elevated temperatures and the values computed for the desired temperature. The vapour pressures were obtained by the linear equation.

$$\text{Log } P = A - B/\pi$$

where $A$ and $B$ are the constants and $\pi$ is the absolute temperature. The constants $A$ and $B$ were determined from the experimental data by the method of least-squares using a computer programme in Basic language. The calculated vapour pressures at 25 °C for all the BMNs are given in Table 2. Seven BMNs compounds having sufficient volatility are presented in Table 2. The remaining BMNs are less volatile.

## 2.3 Determination of Hydrophobicity

The hydrophobicity (log *P)* of each compound was determined using HPLC. A water model ALC-GPC-244, high-performance chromatograph, comprising two model 6000A pumps, a U6K injector, a model 660 solvent programmed and a UV detector (254 nm) was used. The mobile phase consists of

water and methanol in various proportions ranging from 40-70 per cent methanol with a constant flow rate of 1 ml/min. $C_{18}$ ODS column was used as the stationary phase. The capacity factor K for each compound at various solvent compositions was plotted to obtain log K at 100 per cent water, which was used to measure the relative hydrophobicity.

### 2.4 Determination of Depression of Respiratory Rate by 50 per cent

Randomly-bred Swiss male mice (body weight: 27-32 g) maintained in the animal house of the Establishment, were housed in polypropylene cases on dust-free rice husk as the bedding material, and provided food (Amrhut Ltd, India) and water ad *libitum*#. The depression of respiratory rate by 50 per cent ($RD_{50}$) of the exposed animals was determined by the method reported earlier[13].

Briefly, the animals were restrained in a specially designed and fabricated metallic mouseholder for at least one hour before the exposure so as to stabilise the respiratory rate. Thereafter, the mouse holder with the mouse was fitted to an all glass static exposure chamber (capacity: 2 1). The head of the mouse was made to protrude inside the chamber. The inspiration and expiration of the mouse caused a decrease and an increase of air pressure inside the chamber, respectively. These changes were sensed using differential transducer (Grass model PT5), connected through a silicone tube (id, 3 mm) to the chamber. The signals of the respiration, thus generated, were fed to the low-level dc preamplifier of the polygraph (Grass model 7D) and recorded as upward deflection (inspiration) and downward deflection (expiration) on the oscillograph. The respiratory rate was calculated with the help of markings made by the timer (inbuilt) and the deflections made by the respiration.

**Table 2. Vapour pressure, RD, hydrophobicity and K values of various BMNs**

| $R_1$ | $R_2$ | Vapour pressure (25 °C) | $RD_{50}$ (µg/lit) | Log *P* (observed) | Log *P* (calculated) | K value ($s^{-1}$) |
|---|---|---|---|---|---|---|
| H | H | 0.02538 | 73.66 | 1.486 | 2.25 | * 5 X $10^4$ |
| Cl | H | 0.00045 | 6.73 | 2.076 | 2.0 | 20 X $10^4$ |
| F | H | 0.00799 | 13.55 | 0.9300 | 2.32 | * 40 X $10^4$ |
| Br | H | 0.0042046 | 4.86 | 1.650 | 2.35 | 15 x $10^4$ |
| $NO_2$ | H | 0.000055 | 21.5 | 1.106 | 1.90 | 1.0 X $10^4$ |
| OCH3 | H | 0.0068 | > 200 | 1.926 | 2.52 | 1.0 x $10^5$ |
| $CH_3$ | H | Nil | 12.85 | 2.096 | 2.51 | 5 X $10^5$ |
| OH | H | 0.01520 | > 200 | 0.696 | 2.44 | * 18 X $10^5$ |
| H | Cl | 0.00688 | 135.6 | 2.076 | 2.73 | * 10 x $10^4$ |
| H | Br | 0.00056 | > 200 | 1.6500 | 2.62 | 18 X $10^4$ |
| H | $NO_2$ | 0.0025 | 93.55 | 1.106 | 1.65 | * 8 X $10^4$ |
| Cl | Cl | 0.006070 | 48.0 | 2.1050 | 3.10 | 32 X $10^4$ |
| H | OCH3 | 0.000008 | 65.0 | 2.026 | 2.58 | 8 X $10^5$ |
| H | $CH_3$ | 0.0068 | > 200 | 2.026 | 2.51 | 5 X $10^5$ |
| H | OH | 0.00400 | > 200 | 0.696 | 2.20 | 24 X $10^5$ |
| H | $N(CH_3)_2$ | 0.01993217 | > 200 | 1.746 | 2.77 | * 26 X $10^5$ |
| H | COOH | 0.0243449 | > 200 | 3.4400 | 3.10 | * 35 x$10^5$ |

* Compounds having high volatility

# This study has the approval of the Ethical Committee of the Establishment.

*2.4.1 Exposure*

After getting the normal respiratory recording, known quantity of the analogs dissolved in analytical grade acetone was taken (20 µl) in a detachable glass adapter. The adapter was then connected to the chamber and heated using Bunsen flame for 12 ± 2 s (temperature < 300 °C). The generated vapours were purged into the chamber using a rubber bellows fitted with the adapter. For purging the generated vapours, two repeated puffs (20 ml air) by the bellows were given. In all the experiments, the volume of the solvent (acetone) was kept constant (20 µl) as the change in volume might change the respiratory rate. The air concentration was determined by calculating the quantity of the compound present in 20 µl of acetone and the total volume of the exposure chamber (2 l), eg, if 40 µg/l air concentration was required for the exposure, the concentration of the compound in acetone was kept 4 mg/ml. The recordings were done for 5 min and the point of maximum inhibition of respiration was considered for the calculations. At all the connections, atleast three experiments were performed. The analogues of CS show less respiratory depression (eg, if 50 per cent depression was not obtained by 200 µg/l air connection), are shown as $RD_{,,}$ > 200 µg/l in Table 2.

*2.4.2 Analysis of Results*

Fifty per cent depression of the respiratory rate ($RD_{,,}$) was dermined by obtaining the linear curve fitting equation and putting the value of $Y=50$.

## 2.5 Determination of Rate Constants (K Values)

The substituted BMNs induce sensory irritation by reacting with sulphydryl (*SH*) group of receptor protein, which are associated with the nerve endings. For these studies, DEAEM was used as false protein to determine the reactivity of these BMNs. Diethylaminoethyl mercaptan was synthesised as per the method reported in the literature. Studies were carried out at 270 nm in dioxane at 25 °C using Unicam spectrophotometer. For kinetics studies, DEAEM was taken in large excess, wrt the corresponding substituted BMN. The formation of the product was separated and identified using IR and NMR spectroscopic methods and varying rates of reaction of BMNs with *SH* group of the DEAEM were monitored at 270 nm. The reaction was pseudo, first-order, and the K values were calculated from the following equation:

$$K = 2.303 \times l/t \times \log a/a\text{-}x$$

where $a$ is the initial concentration, $t$ is the interval time, and x is the concentration after time $t$.

The results calculated above are summarised in Table 2.

## 3. RESULTS & DISCUSSION

To study the effects of the electron donating and electron withdrawing groups at ortho and para positions of the phenyl ring in the BMNs, attempts have been made to correlate biological activity[14] of various BMNs (Fig. 1) with chemical reactivity towards DEAEM, hydrophobicity, and vapour pressure of these compounds, as shown in the correlation data A and B.

In the present study, it has been observed that the BMNs activate the sensory irritant receptors, both by chemical reaction and physical adsorption. Among all the analogs of BMN studied, CS (2-chloro BMN) followed by 2-bromo BMN are found to be the most active. With the introduction of methoxy, hydroxyl, carboxy group at o/p position of the phenyl ring, desensitisation of the response was observed shortly after the onset of exposure. A similar trend was reported in the literature for substituted aromatic ketones.

## 3.1 Correlation of $RD_{,,}$ Values of BMNs with Chemical Reactivity

Tamtino and Sass[15-16] had compared the chemical reactivity of BMNs with biological activity and had observed a linear relationship between the two with an increase in the number of halogen atoms in phenyl ring. Attempts were made to reinvestigate the correlation between chemical activity and $RD_{,,}$ in both ortho and para series of halogens of various substituted BMNs. Inhalation of a sensory irritant

is directly proportional to the depression of the respiratory rate[17]. Therefore, the depression of respiratory rate is probably the most convenient response to measure[18]. This measurement used to quantify the irritation induced by the inhalation of vapours of the compound". Keeping these facts in mind in the present study, correlation between the chemical activity and the $RD_{50}$ values in both ortho and para series of halogens of various substituted BMNs were also carried out. A good correlation has been found as coefficient of 0.6 was observed in the case of para-substituted halogen while only 0.17 has been found in ortho-substituted ones. Further, it was found that the chemical activity increased by substituting more electron withdrawing groups at the ortho-position. This may be due to the restriction of the free rotation of phenyl group caused by interaction between the ortho substituents and P-hydrogen in BMNs, thus making the P-carbon more prone to nucleophilic attack.

### 3.2 Correlation of $RD_{50}$ Values of BMNs with Vapour Pressure

The vapour pressure of a compound is another physico-chemical property important for the assessment of the irritancy. An attempt was made to correlate the $RD_{50}$ values of substituted BMNs with their vapour pressure[19] data and it was observed that in the case of ortho-substituted BMNs, correlation was only 0.5 while in para substituted BMNs, the correlation was found to be 0.2 only. It may be due to the reason that para-substituted compounds are solids with high melting points and have less vapour pressure, so these have less irritancy than ortho-substituted BMNs. When chemical reactivity and vapour pressure were together correlated with $RD_{50}$ values in both ortho-and para-substituted BMNs, the increase was not significant.

### 3.3 Correlation of $RD_{50}$ Values of BMNs with Hydrophobicity

The relationship between the chemical structures and chromatographic behaviour of chemicals has been discussed*"-*'. However, the exact determination of hydrophobic forces was difficult owing to their complexity. Mayer** and Hemi, and Overton[23], *et al.* used an indirect approach to determine hydrophobicity. They showed a strong relationship between some biological processes and partition of a compound between an organic phase and water, expressed as the partition coefficient. This is the fraction of a dissolved compound that is transferred to the non-polar phase of equal volumes of immiscible solvent. Fujita[24] introduced a substituent constant $\pi$ derived from the partition coefficients, so that

$$\pi = \log \frac{(K/l)X}{(K/l)H} \qquad (1)$$

The partition coefficient is expressed as $K/l$, where $K$ is the fraction in the non-polar phase and $l$ is the fraction in the polar phase; $X$ and $H$ are the substituted and unsubstituted molecules with analogous structures, respectively. A convenient determination of hydrophobicity of compounds is important to study their quantitative structure-activity relationship. The logarithm of the partition coefficient, log $P_{oct}$ of a compound in 1-octanol: Water has been widely used to represent hydrophobicity[21,25]. However, the shake-flask method, generally adopted as standard for the determination of log $P_{oct}$, is not suitable for measuring very high or low partitions and causes variance in the results due to impurities, decomposition, poor detectability and emulsion formation. HPLC has overcome many of these problems being easily amenable to automation. Sabatka,[26] *et al.* used reversed-phase HPLC to measure the hydrophobicity of a series of biphenyl acids and their precursors. Takegoshi[27], *et al.* determined the capacity factor, $K'$ of a series of fungicidal *N*-phenyl carbonates by HPLC with octadecylsilane (ODS) as a stationary phase and a mixture of acetonitrile and water as a mobile phase, using the following Eqn (2):

$$\log K' = \log (t_R - t_o) / t_o \qquad (2)$$

where $t_R$ and $t_o$ are the retention times of the compound and the unretained reference compound, respectively.

In this paper, HPLC method was used to determine the hydrophobicity (log *P)* of the synthesised BMNs from the intercept of the plot (Fig. 2) of log *K'*

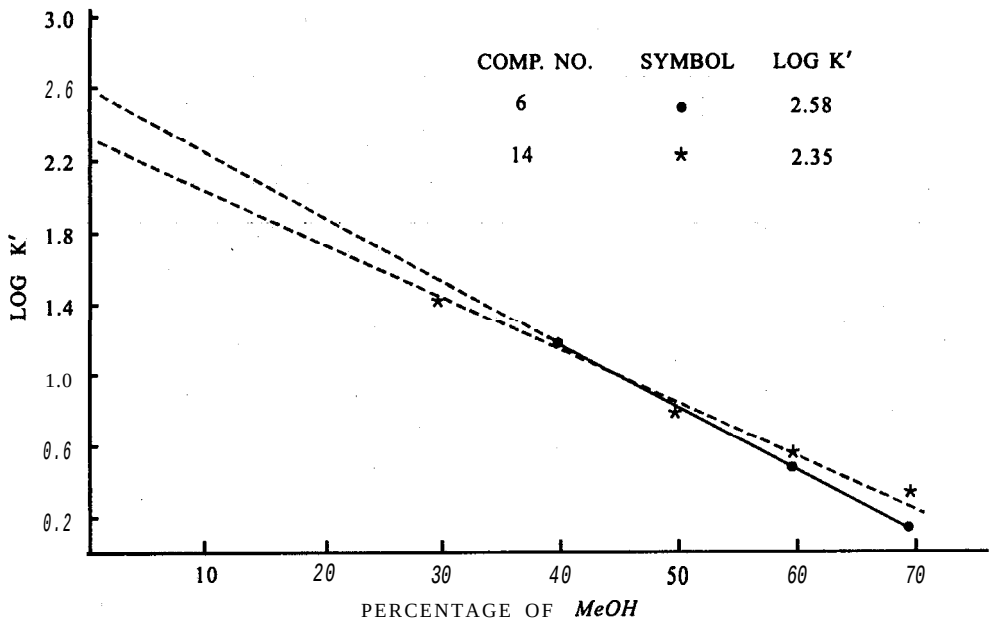



**Figure 2. Reversed-phase HPLC Log *P* values of composition numbers 6 and 14**

versus percentage of *MeOH* in the mobile phase. The results are summarised in Table 2.

The sensory irritancy of the alkyl benzene and ketones increases by increasing the hydrophobicity (lipophilicity) of the molecules[28]. The hydrophobicity depends on the alkyl benzene nucleus with electron withdrawing or electron donating groups. The increase in irritancy of alkyl benzene by increasing the alkyl chain length suggests that the distribution from gas-air phase to a hydrophobic receptor (lip phobic) compartment is responsible for the increase in irritancy. The compounds having moderate log *P* values may be due to the varying rates of reaction of the BMNs with *SH* group of proteins. It was observed that when one halogen atom is present at ortho position in the BMN, the depression in respiratory rate increases. So, halogen atom at ortho position is an important factor to increase the irritancy. The presence of methyl and $OCH_3$ group at ortho position shows less depression in the respiratory rate. This may be due to less influence of $CH_3$, $OCH_3$ groups on the side chain of the molecule. When the changes were made at paraposition of the benzene-ring, the compounds were found to have more hydrophobicity than the unsubstituted BMNs, but showed less irritancy due to the presence of substitution at paraposition. When the log *P* values were correlated with RD,,, only 4.0 and 0.15 correlations were observed in ortho- and para-substituted BMNs, respectively, but when log *P* values were correlated along with K values and vapour pressure (VP) values, then there was a significant increase in the values (0.74 and 0.62 correlation was observed with ortho-and para-substituted BMNs, respectively).

### 3.4 Correlation of RD,, Values of the BMNs with Topological Size of the Molecules

Another factor, which is responsible for the biological activity, is the topological size ($X_o$) of the molecules. The maximum response of the alcohols decreases also by increasing the size (molecular weight, mw) of the alcohols. So, the lo ($X_o$ theoretical values) were also correlated along with K, vapour pressure and log-HPLC values, and a significant increase in the correlation was observed[29]. Correlated values 0.78 and 0.87 were observed in ortho-and para-substituted BMNs, respectively as shown in the correlation data A and B.

***Correlation Data A***

Correlation data of RD,, values with vapour pressure, hydrophobicity, size of molecules, and

chemical reactivity of ortho-substituted BMNs using multiple linear regression equation are:

RD,, vs K = -0.017

RD,, vs VP,, = -0.738

RD,, vs log K = -0.535

RD,, vs log HPLC = -0.408

RD,, vs $X_o$ = 0.116

log P vs log PLC = -0.083

$RD_{50}$ = K + VP,,
$\rho$ = 0.7400
$r^2$ = 0.5500
F = 3.0567

$RD_{50}$ = K + VP,, + log PLC
$\rho$ = 0.7422
$r^2$ = 0.5509
F = 1.6357

$RD_{50}$ = K + $VP_{25}$ + log PLC + Xo
$\rho$ = 0.7575
$r^2$ = 0.5737
F = 1.009

*Correlation data B*

Correlation data of $RD_{50}$ values with vapour pressure, hydrophobicity, size of molecules, and chemical reactivity of para-substituted BMNs using multiple linear regression equation.

RD,, vs K = -0.376

RD,, vs log P = 0.067

RD,, vs log PLC = 0.153

VP,, vs RD,, = 0.174

Iog P vs log PLC = 0.775

RD,, vs Xo = 0.656

$RD_{50}$ = K + VP,, + log HPLC + Xo
$\rho$ = 0.8180
$r^2$ = 0.6693
F = 2.0235

$RD_{50}$ = K + VP,,
$\rho$ = 0.3772
$r^2$ = 0.1423
F = 0.4977

RD,, = K + log HPLC
$\rho$ = 0.3869
$r^2$ = 0.1497
F = 0.5281

RD,, = K + VP25 + log PLC
$\rho$ = 0.3838
$r^2$ = 0.1497
F = 0.2935

where $\rho$ is the correlation coefficient.

## 4. CONCLUSION

From the correlation data, it is clear that sensory irritancy of the BMNs is dependent on all the sections like chemical reactivity, vapour pressure, hydrophobicity, and the size of the molecules, and not dependent on a single parameter such as hydrophobicity, vapour pressure, or chemical reactivity towards DEAEM. A biosignificant correlation (0.75-0.80) was observed between biological activity and physical parameters such as hydrophobicity, vapour pressure, size of molecules, and chemical reactivity of the BMNs with dimethylaminoethyl mercaptans as a simulant of protein–*SH* group using multiple linear regression equation.

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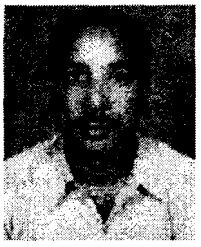

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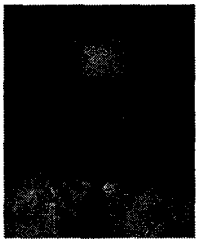

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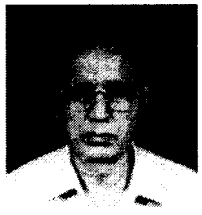

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