CRYSTAL STRUCTURE OF (1*R*,2*S*)-1,2-BIS(4-CHLOROPHENYL)-3,8-DIMETHOXYACENAPHTHENE-1,2-DIOL: TETRAMERIC STRING OF FOUR CONFORMERS CONNECTED BY CLASSICAL HYDROGEN BONDS AND MOLECULAR ACCUMULATION ALIGNMENT BY LINKING OF THE TETRAMERS WITH THE AID OF NON-CLASSICAL HYDROGEN BONDS

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Keywords: S-shaped tetramer composed of four conformers, pseudo-centrosymmetricity, classical hydrogen bonds, non-classical hydrogen bonds

Crystal structure of (1*R*,2*S*)-1,2-bis(4-chlorophenyl)-3,8-dimethoxyacenaphthene-1,2-diol, $C_{26}H_{20}O_4Cl_2$, is reported and discussed from the viewpoints of characteristics in spatial organization, *i.e.*, single molecular structures of four conformers, tetrameric aggregates, and higher ordered structures, with clarification of a classical and two non-classical hydrogen bonding interactions as the structure determining interactions. The title compound crystallizes with four independent molecules (conformers G, B, R, and Y) in the asymmetric unit. Furthermore, each independent molecule displays a *meso* configuration, with one 4-chlorophenyl group *R* and the other *S*. The four molecules are related by inversion center in the asymmetric unit of *P*-1 space group, exhibiting the number of molecules is eight, $Z = 8$. Single molecular structure of each conformer shows that the two benzene rings are bonded with large dihedral angles against the naphthalene plane and the two phenyl rings are oriented in the same direction with respect to the naphthalene ring plane (*syn*-orientation). The four conformers are classified into two groups according to overlapping feature of phenyl rings, *i.e.*, conformers G and Y have larger slippage of the phenyl rings than conformers B and R. In the molecular packing, four conformers Y, B, R, and G are connected by classical O–H…O(H) hydrogen bonds in head-to-head fashion forming S-shaped tetramer. Tetramers composed of four conformers are stacked into columnar structure along *a*-axis through non-classical C–H…Cl hydrogen bonds between conformers G. The columns are linked into a sheet structure by non-classical C–H…Cl hydrogen bonds between conformers G and Y along *ab*-diagonal. The waved sheets are interlocked by two types of non-classical C–H…π hydrogen bonds forming the stripe structure along *c*-axis, *i.e.*, non-classical C–H…π hydrogen bonds between conformers R and Y, and those between conformers B and G.

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Introduction

Understanding of the nature of non-covalent bonding interactions is of great value in chemistry. As the representative non-covalent bonding interactions, classical hydrogen bonds and π…π stacking interactions have been regarded to play a decisive role in crystal structural motif and have been investigated in detail for a long time.[1-6] Accumulation of X-ray crystal structure data has highlighted the importance of next weaker non-covalent bonding interaction, such as non-classical hydrogen bonds where C–H group acts as hydrogen donors, which is especially emphasized in crystal engineering and supramolecular architecture.[7-10] On the other hand, the investigation of weak non-covalent bonding interactions is obliged to be little accounted due to technical limitation in analysis. Contribution of non-classical hydrogen bonds is generally hidden by classical hydrogen bonds and π…π stacking interactions in organic crystals.

The authors envisioned that non-coplanarly accumulated aromatic rings molecules are suitable frameworks for analysing weak non-covalent bonding interactions, because the largely congested molecular circumstances presumably disturb formation of π…π stacking interactions. The authors' recent work has focused on *peri*-aroylnaphthalene compounds and the homologous/analogous substances.[11-20] According to the X-ray crystal structural analyses of ninety *peri*-aroylnaphthalene compounds, the two aroyl groups are non-coplanarly situated to the naphthalene ring and ordinary oriented in an opposite direction (*anti*-orientation). The molecular packing of *peri*-aroylnaphthalene compounds are mainly stabilized by cooperation of several kinds of weak non-covalent-bonding interactions, *i.e.*, four kinds of non-classical hydrogen bonds, (sp^2)C–H···O=C hydrogen bond, (sp^3)C–H···O=C hydrogen bond, (sp^3)C–H···OR hydrogen bond, and C–H···π hydrogen-bonding interaction, and π···π stacking interaction are observed in decreasing order of frequency.[13] As a natural extension of a part of the authors' structural study of sterically crowded 1,8-disubstituted naphthalene compounds, the corresponding reduced derivatives of 1,2-diarylated acenaphthene-1,2-diol compounds are undertaken. Two phenyl rings are connected to sp^2 carbon of carbonyl group in *peri*-aroylnaphthalene compounds, whereas two phenyl rings in 1,2-diarylated acenaphthene-1,2-diol are directly connected to sp^3 carbons of the acenaphthene framework. Such situation means good

opportunity to reveal the hitherto unknown interactions that determine the structure of aromatic rings accumulated molecules in crystalline state.

Herein, the authors report the crystal structure of (1*R*,2*S*)-1,2-bis(4-chlorophenyl)-3,8-dimethoxyacenaphthene-1,2-diol,[21] and discuss correlation among structural features of molecular spatial organization, non-covalent bonding interactions, and molecular packing structure.

Experimental

Materials and methods

All reagents were of commercial quality and were used as received. Solvents were dried and purified using standard procedures.[22] Synthetic methods and spectral data for the precursor, 1,8-bis(4-chlorobenzoyl)-2,7-dimethoxynaphthalene, have been reported in literature.[11, 12, 20]

Measurements

^{1}H NMR spectra were recorded on a JEOL JNM-AL300 spectrometer (300 MHz). Chemical shifts are expressed in ppm relative to internal standard of Me_4Si (δ 0.00). ^{13}C NMR spectra were recorded on a JEOL JNM-AL300 spectrometer (75 MHz). Chemical shifts are expressed in ppm relative to internal standard of $CDCl_3$ (δ 77.0). IR spectra were recorded on a JASCO FT/IR-4100 spectrometer (KBr tablet). High-resolution FAB mass spectra were recorded on a JEOL MStation (MS700) ion trap mass spectrometer in positive ion mode.

X-ray crystallography

For the crystal structure determination, the single-crystal of title compound was used for data collection on a four-circle Rigaku RAXIS RAPID diffractometer (equipped with a two-dimensional area IP detector). The graphite-monochromated Cu Kα radiation (λ = 1.54187 Å) was used for data collection. The lattice parameters were determined by the least-squares methods on the basis of all reflections with $F^2>2\sigma(F^2)$. Crystal data, data collection and structure refinement details are summarized in Table 1. All H atoms could be located in difference Fourier maps, but were subsequently refined in optimized positions as riding atoms, with C–H = 0.95 (aromatic) and 0.98 (methyl) and with U_{iso}(H) = 1.2 U_{eq}(C). For data collection: *PROCESS-AUTO*[23]; cell refinement: *PROCESS-AUTO*[23]; data reduction: *CrystalStructure*[24]; program(s) used to solve structure: *SIR2004*[25]; program(s) used to refine structure: *SHELXL97*[26]; molecular graphics: *ORTEPIII*[27]. The hydrogen bond geometries of title compound are listed in Table 2. Molecular structures of four conformers with the atom-labelling scheme are displayed in Figure 1.

Synthesis of (1*R*,2*S*)-1,2-bis(4-chlorophenyl)-3,8-dimethoxyacenaphthene-1,2-diol

To a 10 mL two-necked round-bottomed flask, 1,8-bis(4-chlorobenzoyl)-2,7-dimethoxynaphthalene (87 mg, 0.20 mmol), zinc (93 mg, 1.2 mmol), zinc chloride (27 mg, 0.20 mmol) and NMP (0.40 mL) were stirred at 373 K under nitrogen atmosphere. After stirring for 2 h, the reaction mixture was poured into water (30 mL). The resulting aqueous solution was extracted with ethyl acetate (20 mL × 3). The combined organic extracts were washed with water (20 mL × 3) and brine successively. The organic layer thus obtained was dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give a cake. Then the cake was dissolved in chloroform (2.0 mL) and the solution was added drop-wisely to hexane (200 mL) for reprecipitation. The precipitates were collected by suction filtration (isolated yield 65 %). Colourless platelet single crystals suitable for X-ray diffraction were obtained by crystallization from methanol (52% yield).

^{1}H NMR δ (300 MHz, $CDCl_3$) : 3.77 (6H, s), 4.37 (2H, s), 6.55–6.85 (4H, br), 6.90 (4H, d, *J* = 9.0 Hz), 7.22 (2H, d, *J* = 9.0 Hz), 7.85 (2H, d, *J* = 9.0 Hz) ppm; ^{13}C NMR δ (75 MHz,$CDCl_3$) : 56.062, 88.320, 114.06, 121.70, 125.11, 126.97, 127.74, 128.06, 132.19, 140.10, 140.87, 153.60 ppm; IR (KBr) : 3420 (–OH), 1627, 1503 (Ar, naphthalene), 1263, 1047 (C–O–C), 979, 822 (C–Cl) cm^{-1}. HRMS (m/z): $[M+Na]^+$ calcd for $C_{26}H_{20}Cl_2O_4Na$ 489.0636, found 489.0677, m.p. = 485–487 K.

Table 1. Crystallographic data and structure refinement parameters of title compound.

Crystal data	
Chemical formula	$C_{26}H_{20}Cl_2O_4$
M_r	467.32
Crystal shape, colour	Plate, Colourless
Crystal system, space group	Triclinic, *P*-1
Temperature (K)	193
a, *b*, *c* (Å)	12.3675(2), 15.9456(3), 23.4719(4)
α, β, γ (°)	73.689(1), 85.136(1), 86.506(1)
V (Å^3)	4423.19(14)
Z	8
Radiation type	Cu Kα
μ (mm^{-1})	2.90
Crystal size (mm)	0.40 x 0.20 x 0.15
Data collection	
Diffractometer	Rigaku R-AXIS RAPID diffractometer
Absorption correction	Numerical NUMABS
T_{min}, T_{max}	0.371, 0.647
No. of measured, independent and observed [I > 2σ(I)] reflections	69658, 15844, 8863
R_{int}	0.051
(sin θ/λ)max (Å^{-1})	0.602
Refinement	
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.059, 0.218, 1.13
No. of reflections	15844
No. of parameters	1178
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.34, -0.54
CCDC no.	1551066

Computer programs: *PROCESS-AUTO* (Rigaku, 1998), *PROCESS-AUTO* (Rigaku, 1998, CrystalStructure (Rigaku, 2007), *SIR2004* (Burla *et al.*, 2007), *SHELXL97* (Sheldrick, 2008), *ORTEPIII* (Burnett & Johnson, 1996).

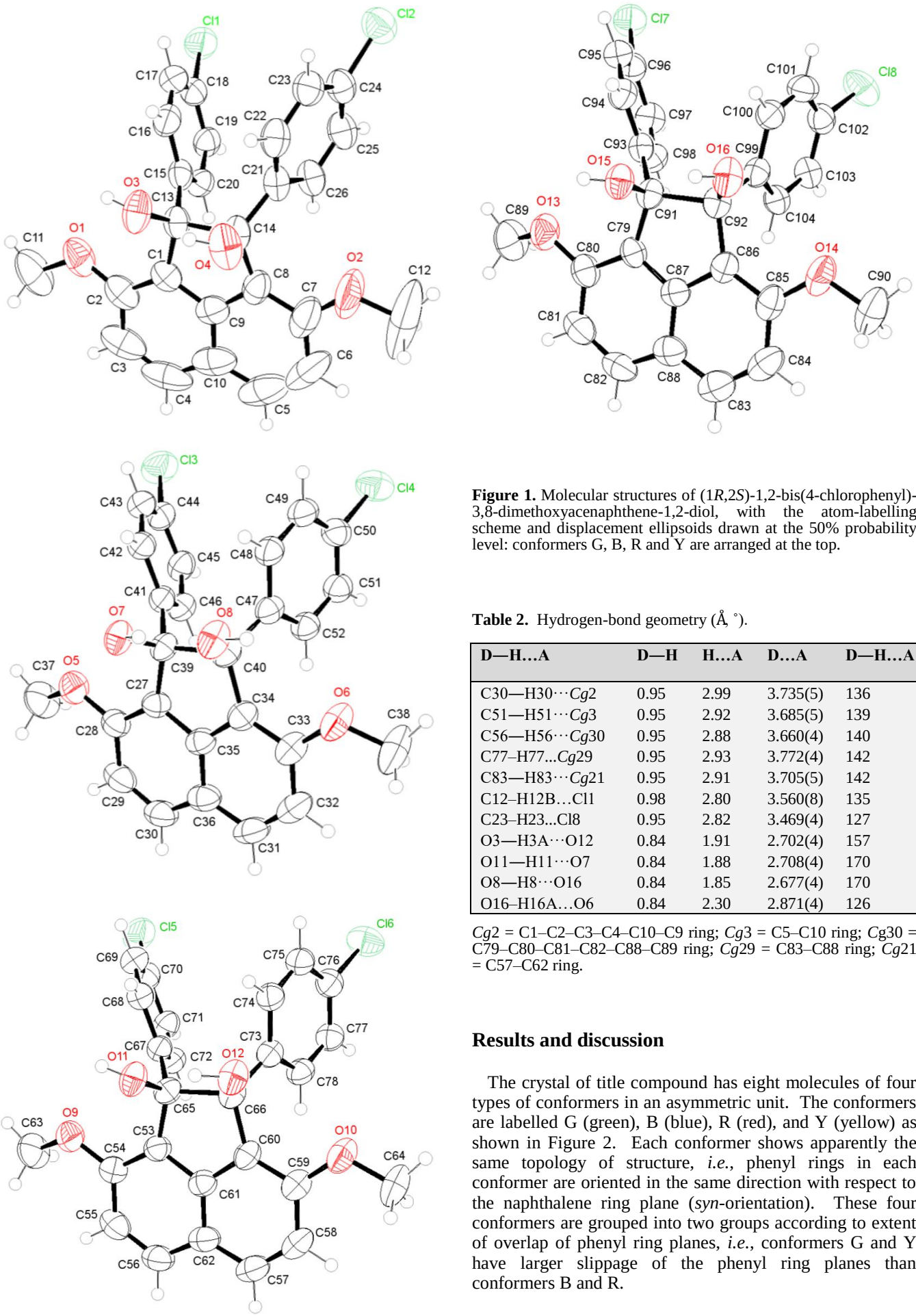


Figure 1. Molecular structures of (1*R*,2*S*)-1,2-bis(4-chlorophenyl)-3,8-dimethoxyacenaphthene-1,2-diol, with the atom-labelling scheme and displacement ellipsoids drawn at the 50% probability level: conformers G, B, R and Y are arranged at the top.

Table 2. Hydrogen-bond geometry (Å, °).

D—H…A	D—H	H…A	D…A	D—H…A
C30—H30···*Cg*2	0.95	2.99	3.735(5)	136
C51—H51···*Cg*3	0.95	2.92	3.685(5)	139
C56—H56···*Cg*30	0.95	2.88	3.660(4)	140
C77–H77...*Cg*29	0.95	2.93	3.772(4)	142
C83—H83···*Cg*21	0.95	2.91	3.705(5)	142
C12–H12B…Cl1	0.98	2.80	3.560(8)	135
C23–H23...Cl8	0.95	2.82	3.469(4)	127
O3—H3A···O12	0.84	1.91	2.702(4)	157
O11—H11···O7	0.84	1.88	2.708(4)	170
O8—H8···O16	0.84	1.85	2.677(4)	170
O16–H16A…O6	0.84	2.30	2.871(4)	126

*Cg*2 = C1–C2–C3–C4–C10–C9 ring; *Cg*3 = C5–C10 ring; *Cg*30 = C79–C80–C81–C82–C88–C89 ring; *Cg*29 = C83–C88 ring; *Cg*21 = C57–C62 ring.

Results and discussion

The crystal of title compound has eight molecules of four types of conformers in an asymmetric unit. The conformers are labelled G (green), B (blue), R (red), and Y (yellow) as shown in Figure 2. Each conformer shows apparently the same topology of structure, *i.e.*, phenyl rings in each conformer are oriented in the same direction with respect to the naphthalene ring plane (*syn*-orientation). These four conformers are grouped into two groups according to extent of overlap of phenyl ring planes, *i.e.*, conformers G and Y have larger slippage of the phenyl ring planes than conformers B and R.

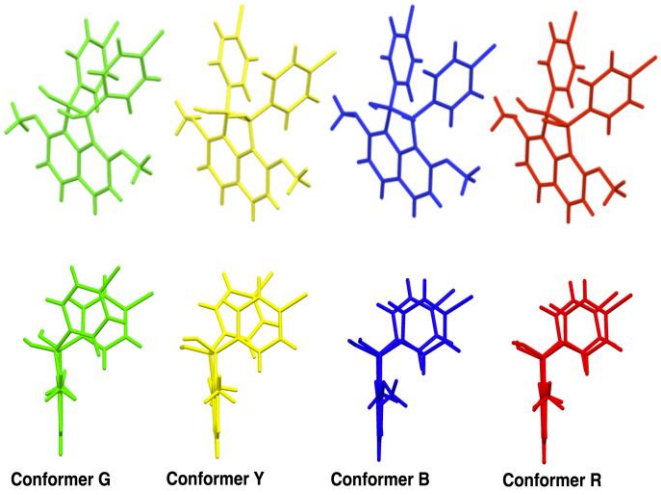


Figure 2. Four conformers classified into two groups by slippage of two phenyl rings: conformers G and Y, and conformers B and R

The respective dihedral angles between the phenyl rings in the four conformers are 36.4 (2)˚ for conformer G, 36.75 (19)˚ for conformer B, 39.26(19)° for conformer Y, and 41.13(19)˚ for conformer R. Dihedral angles between two phenyl rings and the naphthalene ring are 71.71(16) and 76.25(16)˚ for conformer B, 72.04(15) and 72.03(15)° for conformer R, 75.05(17) and 78.89(17)° for conformer Y, and 83.45(19) and 69.79(19)˚ for conformer G. Bond lengths of bridged C–C moiety in four conformers are longer than typical (sp^3)C–C(sp^3) bond. The bond lengths are classified into two groups, *i.e.*, 1.628 Å for conformer Y and 1.634 Å for conformer G, and 1.641 Å for conformer R and 1.642 Å for conformer B. The respective dihedral angles between naphthalene rings and five-membered rings are larger in order of 1.36(17)˚ for conformer B, 1.66(16)˚ for conformer R, 3.66(17)° for conformer Y, and 4.3(2)˚ for conformer G.

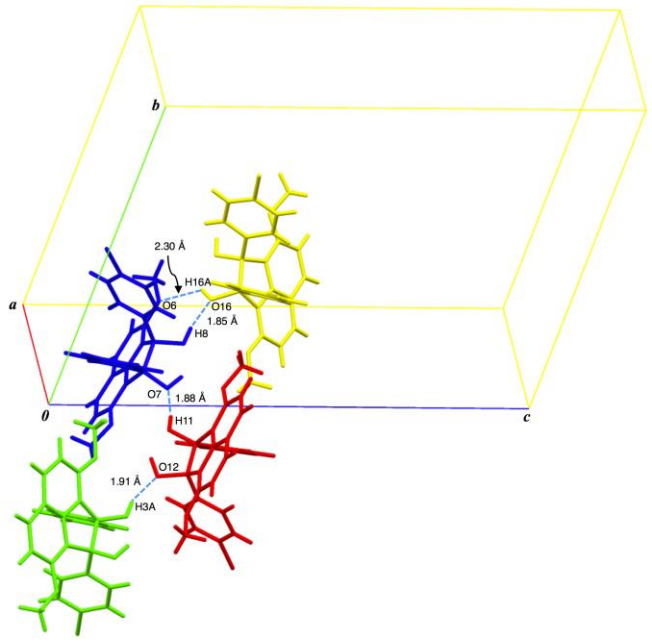
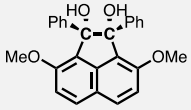
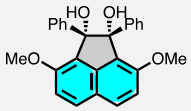
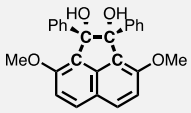


Figure 3. S-shaped tetramer composed four conformers Y, B, R and G.

Table 3. Single molecular structure data of conformers.

Dihedral angles between phenyl rings	
	{G}36.4 (2)˚
	{B}36.75 (19)˚
	{Y}39.26 (19)˚
	{R}41.13 (19)˚
Dihedral angles between phenyl ring and naphthalene	
	{B}71.71(16), 76.25(16)˚
	{R}72.04(15), 72.03(15)˚
	{Y}75.05(17), 78.89(17)˚
	{G}83.45(19), 69.79(19)˚
Bond lengths of bridged C–C bonds	
	{Y}1.628 Å
	{G}1.634 Å
	{R}1.641 Å
	{B}1.642 Å
Dihedral angles between five-membered ring and naphthalene	
	{B}1.36(17)˚
	{R}1.66(16)˚
	{Y}3.66(17)˚
	{G}4.3(2)˚
Torsion angles formed by bridged C–C–C–C bonds	
	{B}3.9(3)˚
	{R}3.9(3)˚
	{Y}11.4(3)˚
	{G}12.8(4)˚

The torsion angles made by three bonds containing two carbons at 1- and 2-positions of the acenaphthene unit are 3.9(3)˚ for conformer B [C27–C39–C40–C34], 3.9(3)˚ for conformer R [C53–C65–C66–C60], 11.4(3)˚ for conformer Y [C79–C91–C92–C86], and 12.8(4)˚ for conformer G [C1–C13–C14–C8].

The structural data described above are summarized as Table 3. These single molecular structure data indicate that slippage of phenyl rings is related with distortion of five-membered ring moiety of the acenaphthene core.

In crystal packing, four conformers Y, B, R and G are connected by classical O–H…O hydrogen bonds in head-to-head fashion forming a S-shaped tetramer (Figure 3). Conformers Y and B are connected to each other through two kinds of classical O–H…O hydrogen bonds [{B}O–H…OH{Y} (O8–H8…O16 = 1.85 Å) and {Y}O–H…OMe{B} (O16–H16A…O6 = 2.30 Å)].

Conformer B is linked with conformer R by classical O–H…OH hydrogen bond [{R}O–H…OH{B} hydrogen bonds (O11–H11…O7 = 1.88 Å)]. Conformers R and G are connected to each other by classical O–H…OH hydrogen bond [{G}O–H…OH{R} hydrogen bonds (O3–H3A…O12 = 1.91 Å)]. Whilst there is no classical O–H…O(H) hydrogen bonds between conformers G and Y. Tetramers composed of four conformers are stacked into columnar structure along *a*-axis through non-classical C–H…Cl hydrogen bonds between conformers G [{G}(methoxy)C–H…Cl{G} (C12–H12B…Cl1 = 2.80 Å)] (Figure 4).

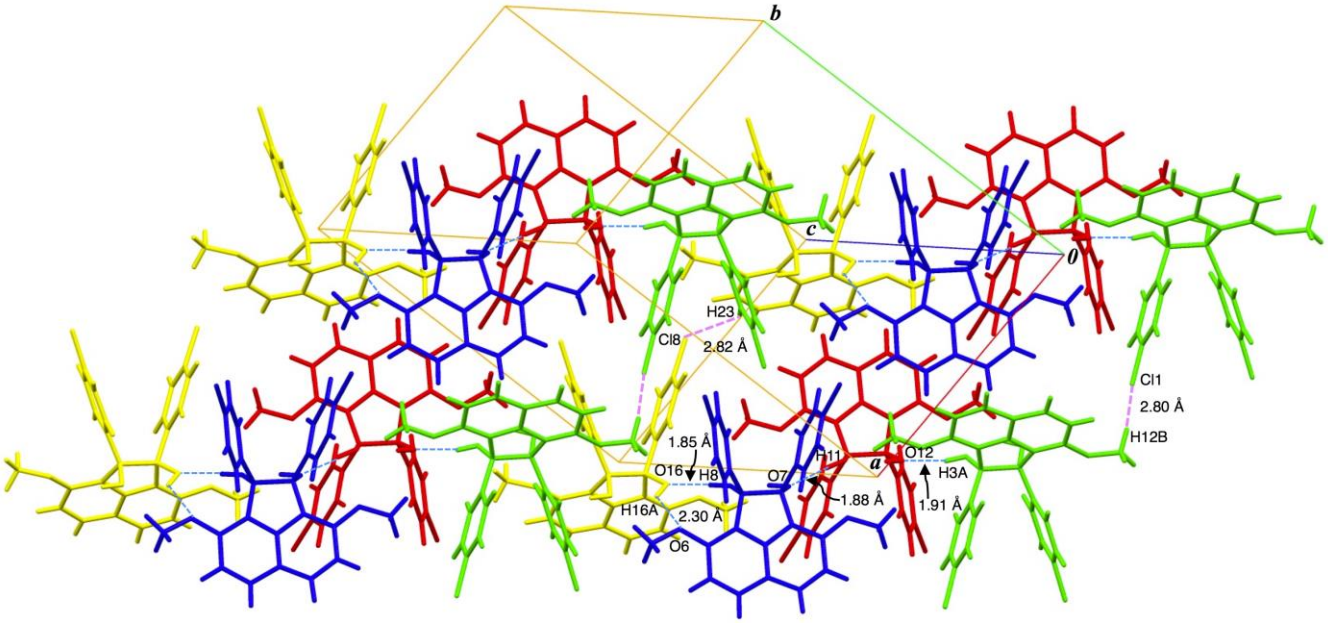


Figure 4. Waved sheet structure of tetramers formed by two types of non-classical C–H…Cl hydrogen bonds (dashed pink lines).

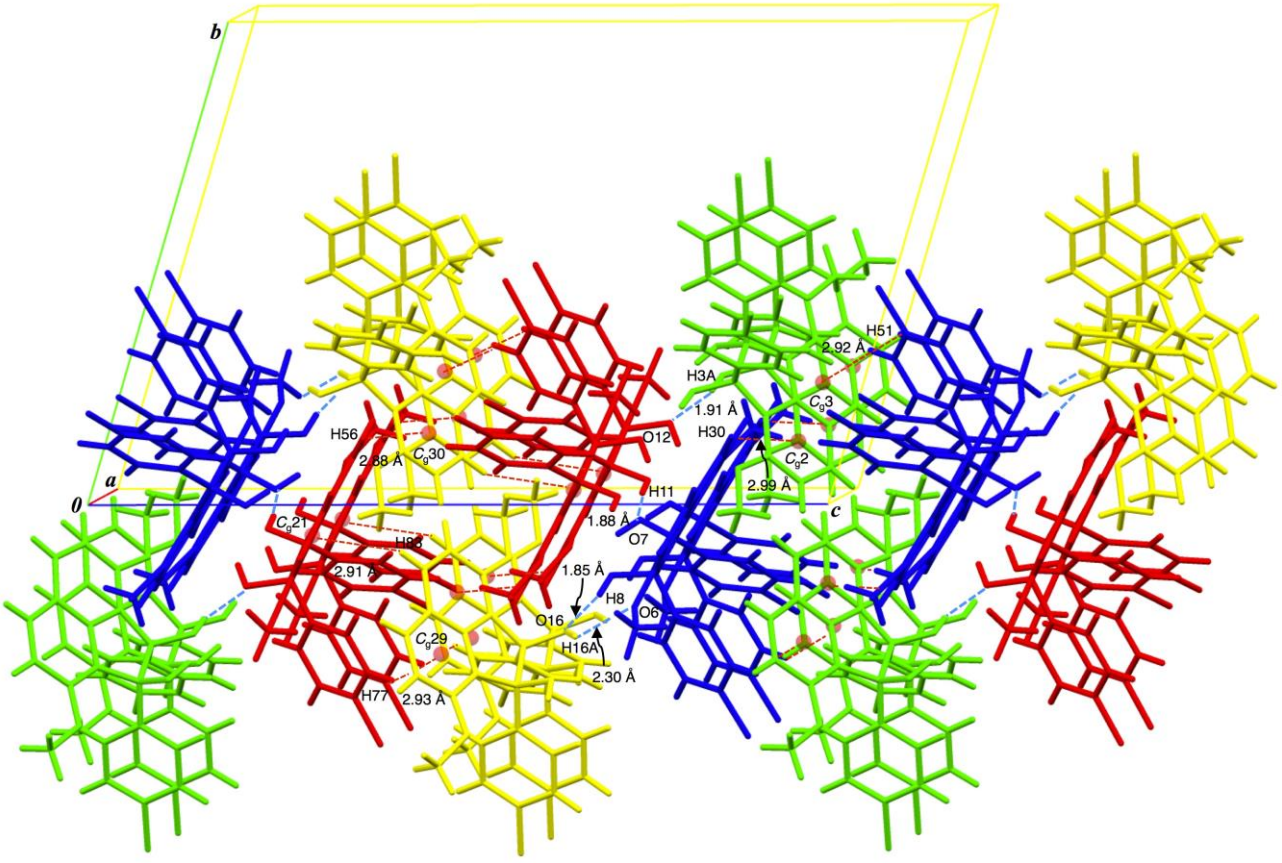


Figure 5. Interlocked waved sheets by non-classical C–H…π hydrogen bonds between conformers R and Y, and those between conformers B and G (dashed red lines).

The columns are linked into a sheet structure by non-classical C–H…Cl hydrogen bonds between conformers G and Y along *ab*-diagonal [{G}(benzene)C–H…Cl{Y} (C23–H23…Cl8 = 2.82 Å)] (Figure 4). The waved sheets are interlocked by two types of non-classical C–H…π hydrogen bonds forming stripe structure along *c*-axis, *i.e.*, non-classical C–H…π hydrogen bonds between conformers Y and R, and non-classical C–H…π hydrogen bonds between conformers G and B [{R}(naphthalene)C–H···π(naphthalene){Y} (C56–H56···*Cg*30 = 2.88 Å; *Cg*30 = C79–C80–C81–C82–C88–C89 ring), {Y}(naphthalene)C–H···π(naphthalene){R} (C83—H83···*Cg*21 = 2.91 Å; *Cg*21 = C57–C62 ring) and {R}(benzene)C–H…π(naphthalene){Y} (C77–H77…*Cg*29 = 2.93 Å; *Cg*29 = C83–C88 ring); {B}(benzene)C–H···π(naphthalene){G} (C51–H51···*Cg*3 = 2.92 Å; *Cg*3 = C5–C10 ring) and {B}(benzene)C–H···π(naphthalene){G} (C30–H30···*Cg*2 = 2.99 Å; *Cg*2 = C1–C2–C3–C4–C10–C9 ring)] (Figure 5).

As described in the preceding Results part, the four conformers have common topology that two aryl groups are non-coplanarly situated to the acenaphthene unit and oriented in the same direction against the acenaphthene ring, *i.e.*, *syn*-conformation. Moreover, the four conformers are naturally divided into two pairs (conformers G and Y, and conformers B and R) from the viewpoint of the spatial organizations of the single molecular structures. The two conformers of the same pair have almost the same structural motif. Effective non-covalent bonding interactions extracted in the accumulation structure of conformers are taken into account the standpoint of crystal structure determining factors. First, classical O–H…O(H) hydrogen bonds are observed between conformers R and B, between conformers R and G, and between conformers B and Y. Secondary, non-classical C–H…Cl hydrogen bond between two molecules of conformer G along *a*-axis and that between conformers G and Y along *ab*-diagonal are observed. Three types of non-classical C–H…π hydrogen bonds between conformers R and Y and two types of C–H…π ones between conformers B and G are also observed. These effective hydrogen bonds are arranged in order of strength, *i.e.*, classical O–H…O(H) hydrogen bonds, non-classical C–H…Cl hydrogen bonds, and non-classical C–H…π hydrogen bonds. Based on the interpretation of preferential account of the strongest interaction, four conformers are linked into Y–B–R–G tetramer mother skeleton through classical hydrogen bonds. The central conformers B and R have essentially same spatial organization, and they are located with a pseudo-centrosymmetric center. The circumstances are essentially same for the alignment of conformers Y and G. Since four conformers have *syn*-conformation as common topology, each conformer has no centrosymmetric center in the molecule. In a natural consequence, they exhibit centrosymmetric center between the paired conformers. On the other hand, effective interactions between tetramers are unsymmetrically formed, especially for the interaction participated by conformers G and Y. Conformer G is linked with conformer G in the adjacent tetramer by non-classical C–H…Cl hydrogen bond along *a*-axis, and connected to conformer Y in neighboring tetramer by non-classical C–H…Cl hydrogen bond along *ab*-diagonal. Conformer R forms three types of non-classical C–H…π hydrogen bonds with conformers Y in another tetramers, and conformer B forms two types of non-classical C–H…π hydrogen bonds with conformers G in another adjacent tetramers. The non-classical C–H…Cl hydrogen bonds two-dimensionally arrange the tetramers parallel to *ab*-plane, and weaker non-classical C–H…π hydrogen bonds interlock the waved sheets forming the stripe structure along *c*-axis.

The molecular packing of title compound can be interpreted as follows: Four molecules related by centrosymmetric center are accumulated to form tetramer structure by stronger interactions of classical hydrogen bonding, which plays the prior function for determination of mother unit of the crystal. The tetramer discriminates the molecules into essentially two types of conformers according to the position in the tetramer, that is, the centered two molecules and the terminal ones. Consequently, the tetramer disproportionates to two kinds of conformers. The two kinds of conformers seem to be distinguished as four kinds of conformers by minute differences. On the basis of the existence of pseudo-centrosymmetricity in tetramer unit, a number of weak non-covalent bonding interactions contribute the arrangement of tetramer units forming second-ordered accumulation structure and the third-ordered one according to the strength of individual interaction. Naturally, the most stabilized accumulation crystalline structure needs to be perturbed. As a result, the crystal is composed of two types of conformers, each of which has two conformers having substantially same spatial organization with small differences.

Conclusion

Crystal structure of a reductively intramolecular-coupled product of *peri*-aroylnaphthalene, *meso*-1,2-bis(4-chlorophenyl)-3,8-dimethoxyacenaphthene-1,2-diol, has been determined. In the crystal, there exist independent four different conformers distinguishable on the basis of degree of distortion of five-membered ring in acenaphthene moiety and the dihedral angle between phenyl and acenaphthene rings. Furthermore, each conformer has unsymmetrical structure to give its enantiomer of discriminative chirality. As a result, a unit cell of title compound contains eight molecules. Each molecule of the compound has adjacent two phenyl groups on the 1,2-positions of acenaphthene skeleton on the same side against the acenaphthene ring plane, *i.e.*, *syn*-orientation. In the same manner, adjacent two hydroxy groups at the same carbons are situated in *syn*-orientation on the opposite side against the acenaphthene ring. Though the chemical formula of the compound is displayed of mirror symmetricity, spatially unsymmtrical alignment of substituent makes chiral situation of molecular structure. On the other hand, the four conformers are divided into two pairs based on the structural similarity of molecular spatial organization in crystal. Two conformers are characterized as larger intramolecular overlapping of phenyl rings together with smaller torsion in the fused five-membered ring moiety of acenaphthene region. The other pair of two conformers has the opposite feature. As the molecular accumulation structure, two conformer molecules of larger overlapping of phenyl rings are positioned adjacently and one molecule of the other type of conformer are situated respectively at the

both outer side resulting in formation of S-character like tetramer string bound by classical O-H…O(H) hydrogen bondings. The tetramers are connected with neighboured tetramer with two kinds of non-classical C–H…Cl hydrogen bondings at the terminal conformer molecules leading the waving planar aggregate on the plane parallel with *a*-axis. Furthermore, the planar aggregates are engaged with each other to be stacked along the *c*-axis. One terminal conformer molecule of a tetramer makes non-classical C–H…π hydrogen bonding with a central conformer molecule of the adjacent tetramer to make the intertetramer connection. As described above, three kinds of non-covalent interaction of far different strength among the molecules of title compound in crystal are recognized to play governing factors to construct the higher ordered molecular accumulation structure by alignment of the molecules with different strength according to the spatial direction.

Though title compound has *meso*-form, *syn*-oriented structure prohibits to have centrosymmetric center in the inner side of molecule. In addition, two hydroxy groups are positioned at one side and the two phenyl groups are situated at the opposite side against acenaphthene plane. Such alignment makes the both side of acenaphthene ring largely different chemical environment. As a natural consequence, pseudo-centrosymmtrical dimeric aggregate formed by strong classical O-H…O(H) hydrogen bonding plays a role of coagulation core to be connected with two molecules at the both outer side by also strong classical O-H…OH hydrogen bonding, resulting in formation of tetramer molecular structure of pseudo-centrosymmetricity. The pseudo-centrosymmetric tetramers thus formed are coagulated to each other by rather weak interaction of non-classical hydrogen bonding in centrosymmetric fashion to yield highly ordered molecular aggregate structure. In the higher ordered structure, minimization of crystal energy might be achieved by adjustment the number and position of the non-classical hydrogen bondings to give rather small difference in the atomic alignment. Accordingly, this realizes the differentiation of molecules as four kinds of conformers belonging two types of spatial organization pattern.

Conclusively, the construction of crystal structure of title compound is recognized based on molecular motif of pseudo-centrosymmetric tetramer aggregation of molecules having largely differentiated aromatic faces connected by classical O-H…O(H) hydrogen bonding, where the tetramers accumulate with minimization of crystal energy by non-classical hydrogen bondings with perturbation of spatial organization of component molecules.

Acknowledgements

The authors would express their gratitude to Professor Keiichi Noguchi, Instrumentation Analysis Center, Tokyo University of Agriculture and Technology, for his technical advice. This work was partially supported by the Ogasawara Foundation for the Promotion of Science & Engineering, Tokyo, Japan.

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Received: 31.05.2017.
Accepted: 08.07.2017.

DENSITY OF STATES OF SINGLE-WALLED CARBON NANOTUBES : A COMPUTATIONAL COMPARATIVE STUDY

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Keywords: Density of states, DFT, Comparative study, ATK

This paper reports the density of states (DOS) of chiral (4,1), armchair (4,4), and zigzag ((4,0) single-walled carbon nanotubes (SWCNTs) by using ab-initio Density Functional Theory (DFT). Our simulation results show the distinguishable features of three types of CNTs in terms of density of states (DOS), so that they can be fully exploited in nano-devices. The results are helpful for studying the working principles of the CNT-based electronic devices and designing new ones.

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Introduction

Among one-dimensional nanostructures carbon nanotubes (CNTs)[1,2] are the most explored one and have attracted tremendous interest from both fundamental science and technological perspectives. CNT is a rolled one atom thick layer called graphene, where the length of the tube is larger than the tube diameter.[3] These nanostructures are topologically simple and exhibit a rich variety of intriguing electronic properties such as metallic and semiconducting behaviour.[3,4] Furthermore, these structures are atomically precise, meaning that each carbon atom is still three-fold coordinated without any dangling bonds. CNTs show a wide range of applications in the area of electronic, optics, medical, mechanics, and in many other industrial areas.[5-11] Therefore, much attention has been given to the investigation of their electrical, vibrational and thermal properties of CNTs.[12-16]

The electronic transport properties of two-probe system of heterojunction formed by an (8, 0) CNT and an (8, 0) silicon carbide nanotube (siCNT) has been reported Liu et al.,[17] whereas Anders Blom et al.[18] investigated the InAs p-i-n junction and calculated the transport characteristics of the system using two different approaches. In this work, we calculated the density of states (DOS)of chiral, armchair and zigzag SWCNTs by using density function (DFT) calculation of atomistic toolkit (ATK) software.

Results and Discussions

The carbon–carbon bond lengths of the simulated (4,1), (4,4) and (4,0) carbon nanotube structures are taken as 1.423 Å whereas the lengths of the central regions of the simulated sections are taken as 3 periods. The geometries of the simulated structures are shown in Figure 1a-1c.

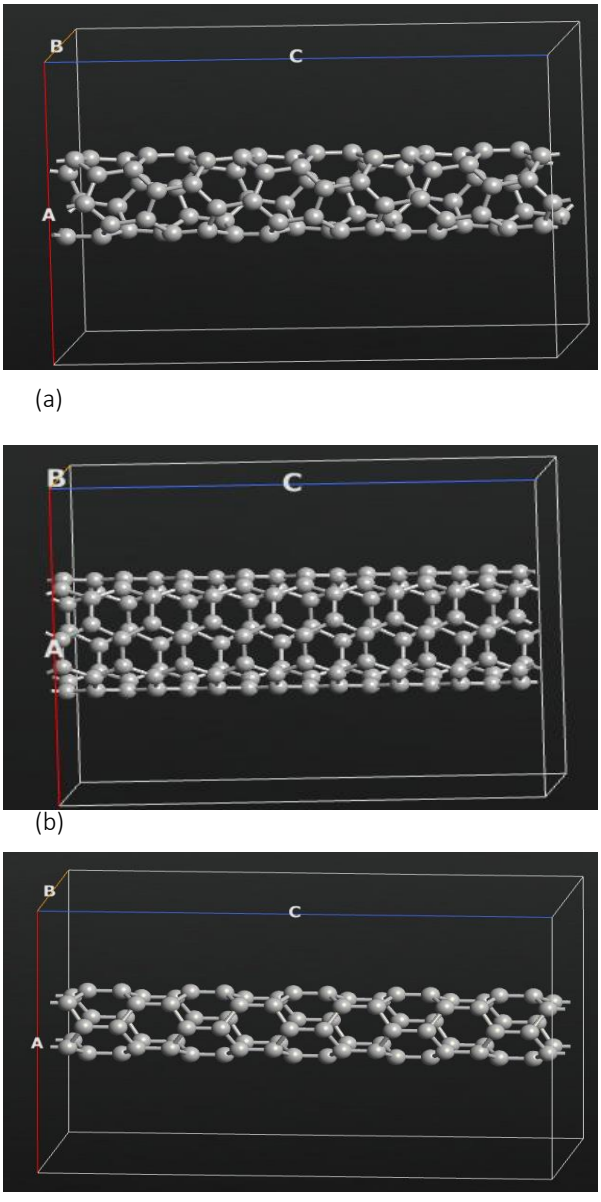
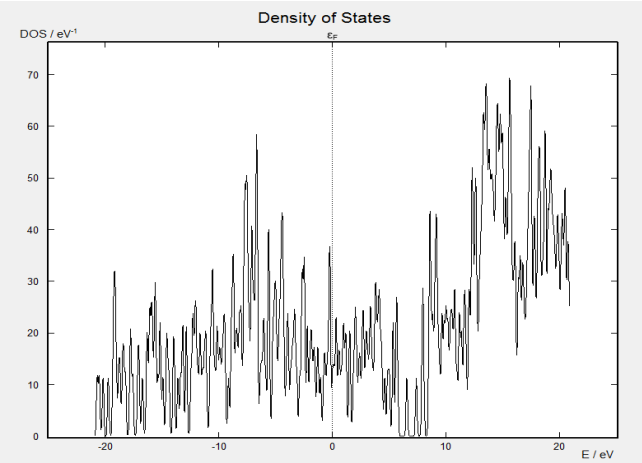
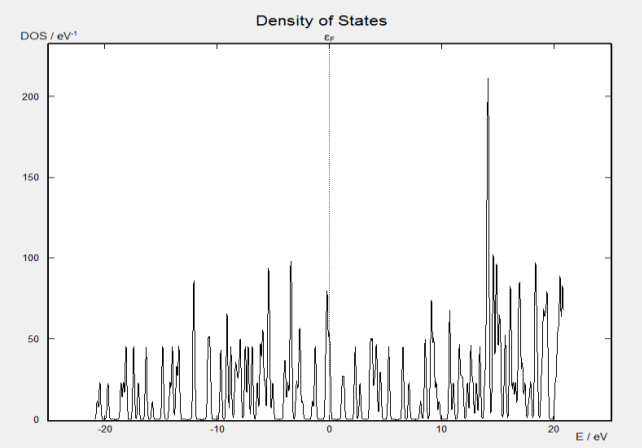


Figure 1. Geometrical structure of (a) (4,1) CNT (b) (4,4) CNT and (c) (4,0) CNT**.**



a)



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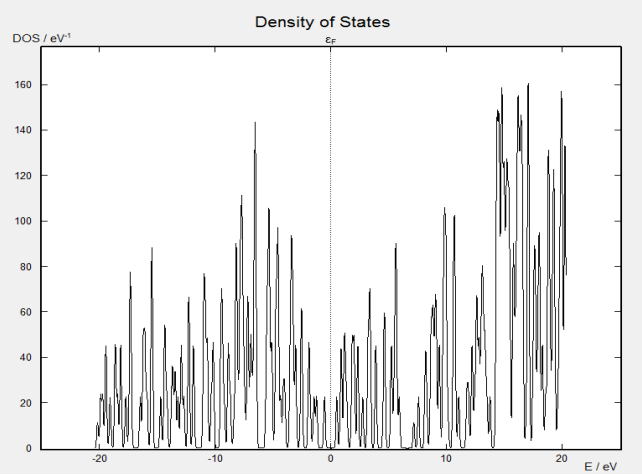

c)

Figure 2. Density of states (DOS) for (a) (4,1) CNT (b) (4,4) CNT and (c) (4,0) CNT.

The DFT simulation parameters are selected to be the following: mesh cut-off energy is taken as 150 Ry, basis set is double zeta polarized with 0.001 Bohr radial sampling. Brillouin zone integration parameters of electrodes are taken as (1,1,1) and electrode temperature 300 K. These parameters are chosen to provide accurate results as reported earlier.[19] In DFT simulations, the electrodes are assumed to be repeated infinitely in the transport direction and to have bulk-like properties. The length of the electrodes is thus chosen to be sufficiently long to ensure that there is no interaction between the central region and the repeated images of the electrodes. The basis sets used the single-zeta polarized (SZP), and the double-zeta polarized (DZP).[20,21] The DZP is the mostly complete basis set we used, and therefore the one that best predicts the ground state of the system.[21]

To understand the localization of electrons near the Fermi level, we have plotted the density of states profile of the three proposed models using the ATK-DFT. [22] ATK-DFT is based on density functional theory and applies a local atomic orbital (LAO) basis set and Perdew, Burke and Enrzenhofer parameterization of generalized gradient approximation (GGA).[23]The DOS profile of chiral (4,1) CNT is shown in Figure 2(a), whereas the DOS profile of armchair (4,4) CNT and zig-zag (4,0) CNT is shown in Figure 2b and Figure 2c respectively. Figure 2 shows that all these three structures have a distinct DOS in longer energy ranges.

Conclusions

In this study, three SWCNT geometries were simulated using DFT of Atomistic Tool Kit (virtual nanolab) to investigate their distinguishable density of states (DOS) in long energy range. The DOS near the Fermi level in case of chiral and armchair nanotubes are very close to each other which is not the case for zig-zag nanotube. Therefore this study clarifies the theoretical aspects of CNTs so that they can be fully explored in the future electronic industry.

Acknowledgements

The author greatly acknowledges the financial support by University Grants Commission (UGC), New Delhi (Grant F-No: 42-768/2013) which enables this study to occur.

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Received: 03.06.2017.
Accepted: 08.07.2017.

ANALYSIS OF THE CORRELATIONS BETWEEN NO, NO_2 AND O_3 CONCENTRATIONS IN CAMPO GRANDE – MS, BRAZIL

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Keywords: Ozone; Nitrogen dioxide; NO_x; Oxidant; Regional Pollution; Local pollution; Nitrous acid; Network monitoring.

Ozone (O_3) is a secondary gaseous pollutant in the urban environment, and its variation correlates well with nitrogen oxides (NO_x = NO + NO_2). Continuous monitoring has been done in the Campo Grande city urban area, using ozone 49C and NO-NO_2-NO_x 42CTL gas analyzers. The results show that the maximum concentrations of O_3 and oxidant (O_x = O_3 + NO_2) in Campo Grande often appear in the early afternoon around 15:00 hours. The daily variation of NO concentrations shows a very clear cycle with two peaks, one appearing around 07:00 a.m. and the other at 11:00 p.m. At the lowest level, NO_2 is the main component of NO_x, while NO dominates the higher mixing ratio. It is also shown that the level of O_x is composed of two factors: the regional and the local contributions. The former is affected by the regional O_3 level, while the latter is effectively correlated with the primary pollution level. The daily variation also appears in the concentrations of oxidant components.

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INTRODUCTION

One of the main problems caused by air pollution in urban areas is the presence of photochemical oxidizers. Among these, ozone (O_3) and nitrogen dioxide (NO_2) are particularly important since they are susceptible of provoking adverse effects on human health (OMS, 2000). The formation of ozone at ground level depends on the intensity of the solar radiation, the absolute concentration of NO_x and the VOCs (Volatile Organic Compounds), and the ratio between NO_x and VOCs.

Various observations have shown that, in shiny days, the ozone concentration increases with the growing intensities of solar radiation and temperature. The concentration of photochemical oxidizers may be reduced throughout the control of theirs precursors, which are nitrogen oxides NOx (NO and NO2) and VOCs.[2-4] It is necessary, hence, to search for a complete comprehension of the relationships involving concentrations of O_3, NO and NO_2 under different atmospheric conditions. Different authors, [5-7] studied the relationship between the environmental levels of O_3, NO and NO_2 to improve the comprehension of their chemical coupling.

There is not an official network for air quality monitoring installed in the city of Campo Grande. Nonetheless, some studies and campaigns of weather and climate monitoring have been systematically realized by different researchers,[8-12] Furthermore, modeling of urban atmospheric dispersion has been applied to exploit results of Campo Grande climate effects. [13-14] It has been developed, recently, the first version of a CO and NO_x inventory of emissions. [8]

In the present study, the values of environmental concentration of O_3, NO, NO_2 and NO_x, continually measured in the city of Campo Grande, are used to investigate, for the first time for this town, the concentration of oxidizers (O_x, O_3 and NO_2) along with NO_x. This analysis shall contribute to a better understanding of atmospheric sources of O_x for this specific urban area. The relationships that were found to describe the concentrations of O_3, NO and NO_2 are in agreement with the current knowledge of their chemical coupling.

MATERIALS AND METHODS

Studied and observational data

Campo Grande is the capital city of South Mato Grosso (MS) state, located in the southern of Brazil Midwest region, sited in the center of the state. Geographically the city is near to the Brazilian border with Paraguay and Bolivia. It is located at 20°26'34'' South latitude and 54°38'47'' West longitude. It occupies a total area of 8,096.051 km² or 3,126 mi², representing 2.26% of the total state area, within 860,000 inhabitants (2016) and a corresponding HDI of 0.78. The urban area is approximately 154.45 km² or 60 mi², where tropical climate and dry seasons predominate, with two defined seasons: warm and humid in summer, and less rainy and mild temperatures in winter. During the months of the winter, the temperature can drop considerably, arriving in certain occasions to the thermal sensation of 0ºC or 32ºF with occasional and light freezing. The year average precipitation is 1,534 millimeters, with small up or down variations. The main pollution problems in the city are attributed to the traffic of vehicles, to the raise of building

activities, to the presence of dumping grounds, to the use of small power generators running on oil to supply the lack of electric grid power, and to the induced fire outbreak used to clean up local terrains.

Ensemble of observational data

The air quality and meteorological variables are monitored by an automatic station operated by the Institute of Physics of the Federal University of South Mato Grosso (UFMS). This station is located on the University campus, 8 km or 5 miles to the west of downtown. The main sources of pollution in that area are the building activities; therefore, there are no significant precursor sources of ozone identified close to the region. The ozone levels of Campo Grande area are stored in a regular database since 2004. The equipment of measurement was installed at the top of a tower from where air samples are extracted throughout vertical pipes that are placed approximately 2 meters above the ground level. The concentrations values for O_3, NO_x, NO and NO_2, the ultraviolet (UV) radiation and other meteorological features, such as the air temperature, relative humidity, wind speed and direction are values measured systematically.

The concentrations of pollutants NO, NO_2, NO_x ($NO+NO_2$) and O_3, were measured continuously during a one–year period (2015). The equipments used for measurements included a nitrogen oxide analyzer (AC31M–using chemiluminescence method), an ozone analyzer (O341M–LCD/UV Photometry). All equipments were made by Environnement S.A.

RESULTS AND DISCUSSIONS

Hourly variation of O_3, NO, NO_2, and NO_x concentrations

The average per day variation observed for the NO, NO_2, NO_x and O_3 concentrations are exhibited in figure 1. The daily cycle of ozone concentration reaches a peak during the middle day and presents smaller concentrations during the night. The ozone concentration slowly increases after the first rays of sun shining, getting to its maximum value during the daylight period, after which it starts to decrease slowly until the next morning.

The variation pattern of the average concentration of gases was well defined during the analyzed period, and the pronounced peak of the concentration of NO in the first hour of the morning was due to photolysis of the nocturnal NO_2 accumulated after sunset ($NO + O_3 \rightarrow NO_2 + O_2$), both for NO and O_3 values are minimal at this time (Figure 01). After sunset, a concentration of NO increases slowly. This occurred because of the variation of the emission of NO due to the change of temperature and humidity[16]. Other mechanisms of heterogeneous reactions during day and night may be important, although few known, such as the influence of humidity, temperature and precipitation[17]. The NO_2 (Figure 1) clearly shows that not only the lowest concentration values occur around midday, but also the lower variability in this time, which can be explained by Figure 02, where they have a mean daily value higher global solar radiation and NO photolysis, which reaches maximum values and lower zenith angle. This causes a greater penetration of the solar rays (<400 nm), responsible for the photolysis of the NO_2, since, due to the strong UV radiation, has high photochemical activity, in addition, there is the later oxidation of NO_2 by part of the hydroxyl radical (OH) ($NO_2 + OH \rightarrow HNO_3$), which is the main mechanism of NOx loss[18].

The levels of NO_2 observed in the atmosphere showed a general tendency in which they tend to decrease in the period of the day of greatest insolation and to grow at the end of the day (Figure 1). This fact suggests that the photochemical processes are the main mechanisms to remove NO_2 from the atmosphere with formation of by-products. The model that best explains this fact is the consumption of NO_2 by direct photochemical reactions generating ozone. The formed O3 reacts with water vapor and generates HO radicals, this radical is the main chemical species involved in the NO_2 consumption, with the production of HNO_3, the reaction of formation of nitric acid is significant in the region under study, mainly due to the conditions Favorable conditions of insolation during a large part of the year, the presence of oxidants in the atmosphere is determinant for the formation of NO_2 in the atmosphere, from the NO emitted by the combustion processes. The production of NO_2 occurs mainly by the reaction of NO with O_3 [19-20].

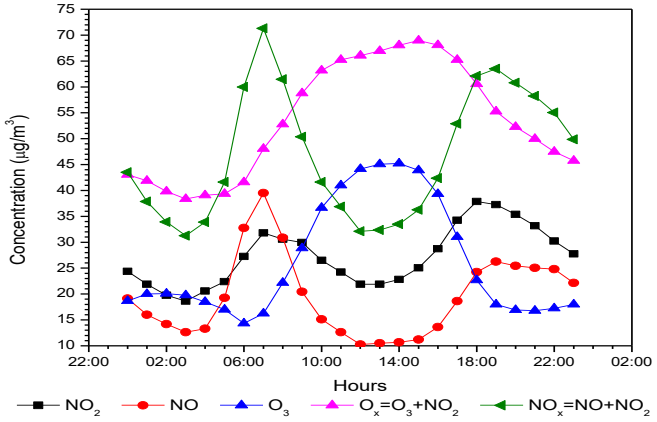


Figure 1. Average of measured values for a daily period of NO, NO_2, NO_x, O_3 and O_x concentrations. The interval between measurements equals 1 hour.

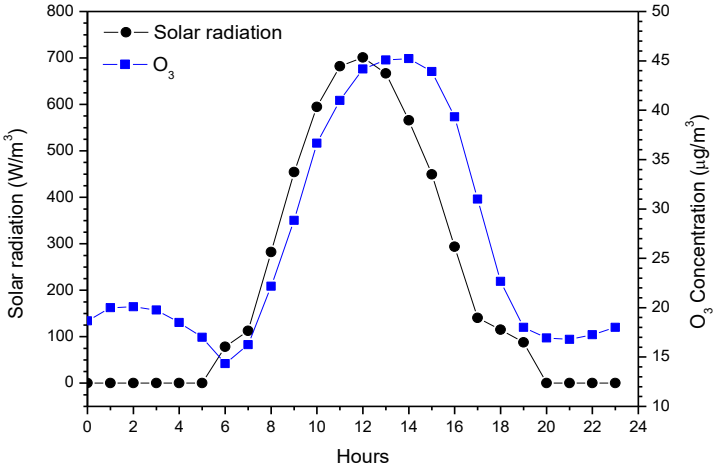


Figure 2. Average concentrations of O3 and UV irradiance for the studied period.

Such a variation is due mainly to the photochemical formation and meteorological conditions. The effect of the increasing solar radiation that occurs between 08:00 and 14:00 / 15:00, along with the height of the mixture layer entails the reduction of NO_x concentration and a raising in the concentration of O_3. The simultaneous measurement of O_3 and UV during the daylight period (from 07:00 to 19:00, see Figure 2) shows that the concentration of O_3 is strongly correlated to the UV irradiance (W/m²). The daily cycles of O_3 and UV flux are similar, with the maximum O_3 occurring at 14:00, i.e., approximately 1 or 2 hours after the maximum UV flux. The statistical analysis reveals that the correlation between O_3 concentration and UV is significant with a correlation coefficient (R) of 0.79.

While O_3 and a large percentage of NO_2 are secondary contaminants, NO is a primary contaminant, formed through a complex set of chemical reactions. At 07:00, the sun light begins to induce a series of photochemical reactions. NO is converted in NO_2 through a reaction with O_3. During the shining hours, NO_2 has converted again into NO because of photolysis, which induces the regeneration of O_3.

Another factor influencing the atmospheric pollutant concentrations is the height of the mixture layer over the city. In a shiny day, pollutants are diluted when the mixture layer increases during the day and stays limited to the inside of NPBL during the night. Emitted pollutants, like NO, are kept underneath such an inversion, and it can cause an increase of the hourly average concentration of NO_x overnight.

Chemistry of O_3, NO and NO_2

The basic chemistry that leads to the production and destruction of ozone has been detailed elsewhere. [26]

$$NO_2 + h\nu \rightarrow NO + O \quad (1)$$

$$O + O_2 \rightarrow O_3 + M \quad (2)$$

$$O_3 + NO \rightarrow NO_2 + O_2 \quad (3)$$

where

M represents a molecule absorbing the excess of vibrational energy and thus stabilizing the O_3 molecule that has been formed, normally it is N_2 or O_2;

hν represents the photon energy, with a 424 nm wavelength; and

O is an active monoatomic molecule of oxygen.

These equations form a cycle free of liquid chemistry, i.e., the global effect of reaction (2) is the opposite of reaction (1). Such reactions represent, therefore, a closed system for which the components NO_x (NO and NO_2) and O_x (O_3 and O_2) are referred separately. Along the daytime, concentrations of NO, NO_2 and O_3 remain typically balanced during a period of a few minutes. Such a situation is known as a photocatalytic state. The concentrations of NO, NO_2 and O_3 are related through the following equation:

$$\frac{[NO][O_3]}{[NO_2]} = \frac{t_2}{k_2}$$

where

t_2 is the NO_2 photolysis rate, and

k_1 is the coefficient of reaction between NO and O_3.

The variation of t_2/k_1 average value along the time was obtained using the observed measurements of NO, NO_2 and O_3 is exhibited in Figure 3. The average value of t_2/k_1 is 16.9, with a minimum of 11.9 and maximum of 22.4 μg m^{-3}. The maximum value occurs typically at 08:00 hours.

The k_1 coefficient varies as a function of temperature (T). Sienfeld and Pandis,[27] proposed the following equation for k_1:

$$k_1\left(1/(ppm\,\min)\right) = 3.23\times10^3 \exp\left(\frac{-1430}{T}\right) \quad (4)$$

As expected, the variation of k_1 is similar to the variation of the average air temperature.

Figure 4 presents the variation of daily concentration of O_3 as a function of the ratio $[NO_2]/[NO]$, sampled hourly. The level of O_3 increases with the raise of $[NO_2]/[NO]$. According to Figure 4, the concentration of O_3 increases rapidly for small values of $[NO_2]/[NO]$. For levels that are more elevated, the concentration of O_3 gets close to reach a photocatalytic state, when it remains relatively stable. In our study, we adjust these data to a polynomial function of $\ln([NO_2]/[NO])$ that can be used to predict the concentration of O_3 during the day:

$$[O] = 20.82 + 14.22 \ln\left[\frac{NO_2}{NO}\right] (R^2 = 38.34\%) \quad (5)$$

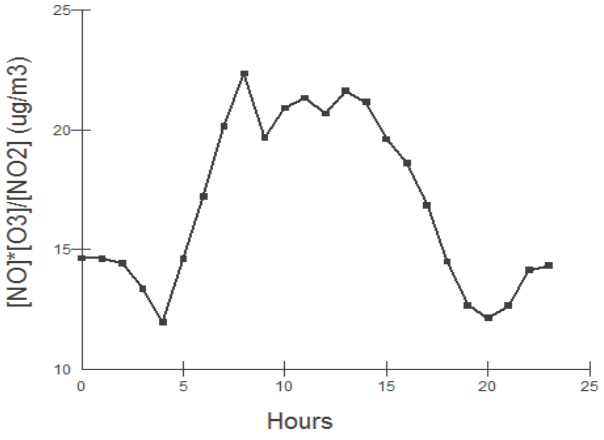


Figure 3. Daily variation of t_2/k_1 (μg m^{-3}) average values

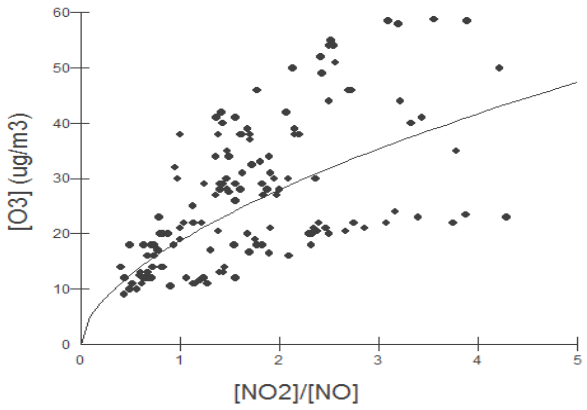
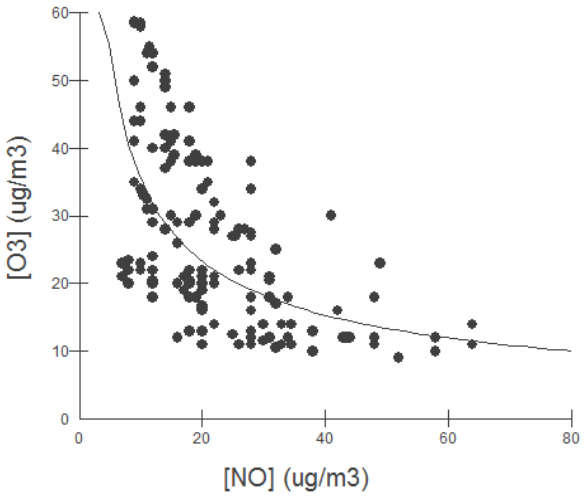


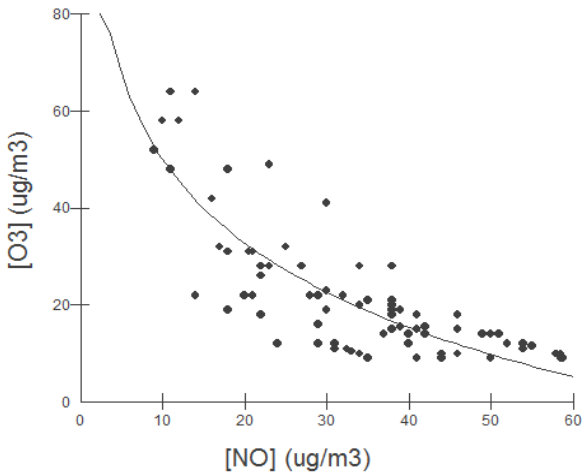
Figure 4. Variation of O_3 as a function of the $[NO_2]/[NO]$ ratio.

The comparison between the average concentrations of NO and O_3 is exposed in Figure 5; the measurements have been taken at one-hour intervals. Three periods have been used for purpose of comparison: the whole day: Y=71.25-15.93ln[NO]; R^2=36.8 %. Daylight (from 06:00 to 18:00): Y=107.98-25.10ln[NO]; R^2=67.96 % and nighttime shift (from 18:00 to 06:00):Y=75.40-18.20ln[NO]; R^2=33.26 %.

(a)



(b)



(c)

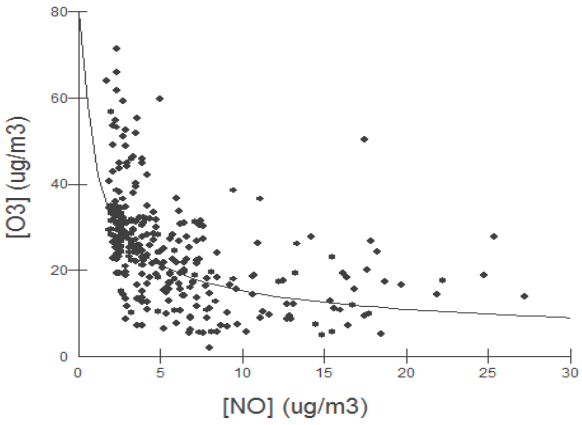


Figure 5. Variation of average values of O_3 plotted along with NO. (a) for the entire day; (b) for the daytime; (c) for the nighttime.

The average concentration of O_3 decreases when the concentration of NO increases. Comparing the values of average concentrations of NO_2, NO_x and O_3, the following characteristics can be observed:

- ✓ The average concentration (1 hour) of O_3 decreases along with the increase of NO_x, while the levels of NO and NO_2 increase with NO_x;
- ✓ The largest concentrations of NO_2, NO and O_3 observed during the day are 48, 64 and 58.8 μg m^{-3}, respectively;
- ✓ The largest concentrations of NO_2, NO and O_3 observed during the night are 49.50, 48 and 39 μg m^{-3}, respectively;
- ✓ The numbers presented on items b) and c) below confirm that the larger daily average concentrations (1 hour) of NO_2 and O_3 are higher during the daylight period than those measured during the night time. However, the highest average (1 hour) concentrations of NO and NO_x observed during the full period considered for this study have occurred during the night time.

Diurnal variation of O_x

The difference between the diurnal and nocturnal O_x behavior shall be expected if the photochemical processes have any influence on the O_x levels in polluted areas. Figure 6 shows the daily variation of the average concentration value of O_x taken at intervals of 1 hour. The concentration of O_x, likewise the variation of O_3 concentration, exhibits a peak at noon and lower concentrations during the night. The concentration of O_x slowly increases after the sun rises, attaining a maximum value during the day and, in the sequence, decreases until the next morning. This is due to the photochemical formation of O_3. Figure 7 shows the daily variation of NO_2/O_x.

The differences on the partition of NO_2 and O_3 can be related to the rate of chemical processes or with the available time for them to occur.

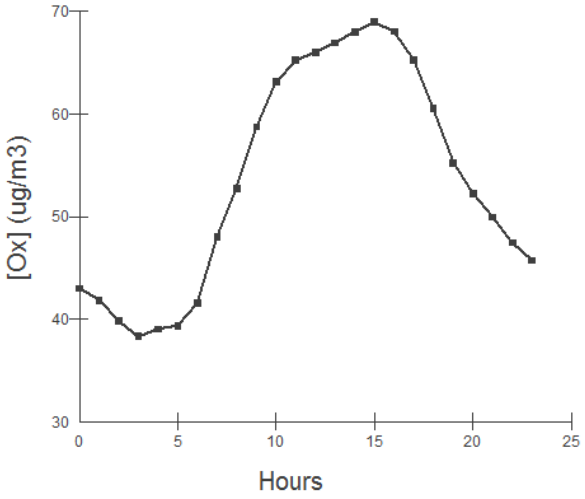


Figure 6. Daily variation of O_x average values.

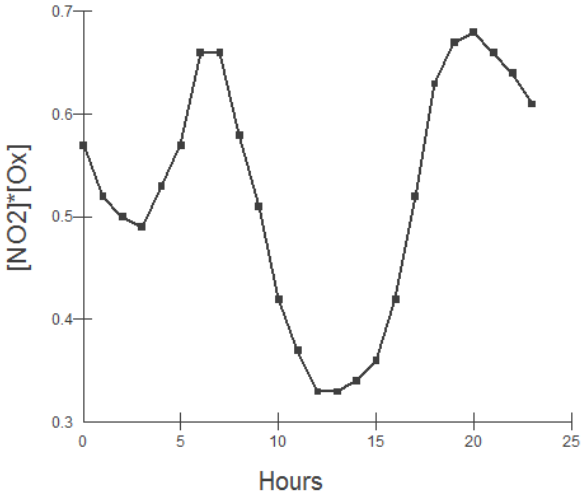


Figure 7. Daily variation of NO_2/O_x average values.

Variation of NO_2/O_x daily values with NO_x

Figure 8 shows the adjusted expression of [NO2] / [OX] as function of [NOx]: Y = 0.71 + 0.33 * ln [NOx]; with a correlatio coefficient of R^2 = 55.2%. The data show that, for lower values o the relation [NO2] / [Ox] there are low NOx values, implying that at these times, Ox concentrations are predominantly marked b high concentrations of O_3. In addition, with increasing NO concentrations, a large part of the concentration is in the form o NO_2. The high values of [NO_2] / [OX] can also be explained by th oxidation process of NO to NO_2 with concentrations of NO marked mainly by NO_2 concentration.

Correlation between concentrations of NO, NO_2 with NO_x

Figure 9 exhibits both variation of NO_x as a function o NO and NO_2 for the average data observed with th sampling interval of 1 hour. The straight lines are, respectively: a) Y=18.54+1.14[NO_2]; R^2=28.44 %; b) Y=28.93+1.07 [NO]; R^2=70.176 %..

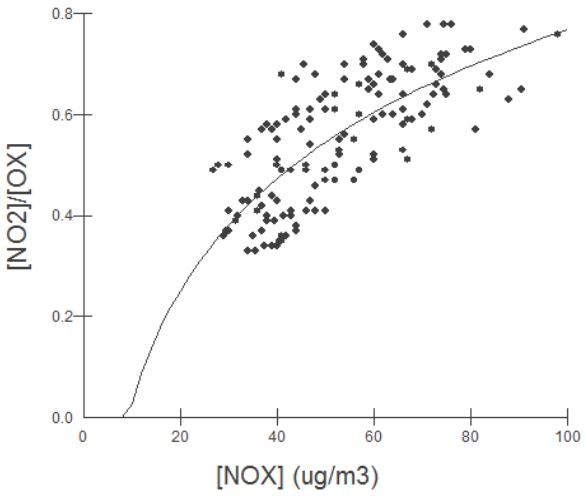
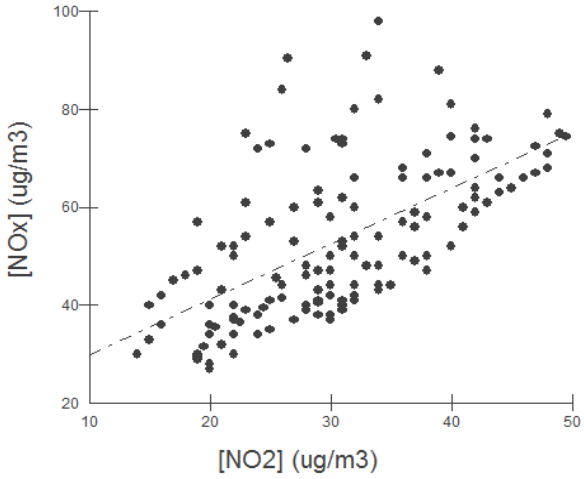


Figure 8. Daily variation of NO_2/O_x average values as a function of NO_x level.

When our data were adjusted to a linear function, the obtained correlation was weak.

(a)



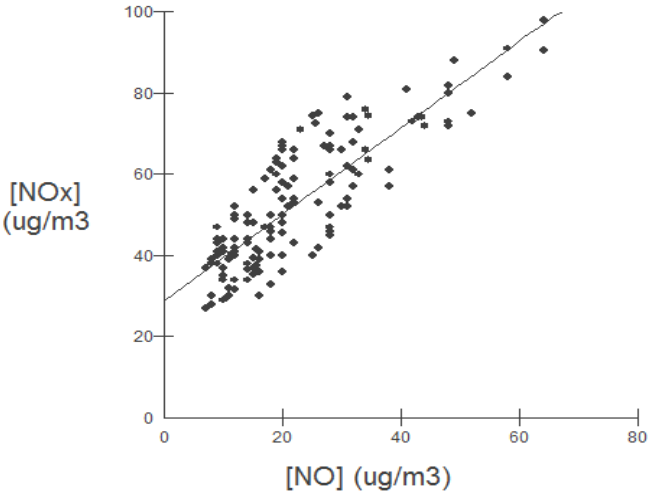


Figure 9. The average values of NO_x plotted as a function of (a) NO_2 and (b) NO.

However, better results were obtained when the observed data are split into diurnal and nocturnal periods. In Figure 11 the observed data values for the diurnal period are exhibited and the respective relations obtained are: a) Y=15.98+1.10[NO_2]; R^2=31 %], and b) Y=29.78+1.06[NO]; R^2=79.1 %. In Figure 12, the measured data for the nocturnal period are exhibited and the relations obtained are: a) Y=20.37+1.09[NO_2]; R^2=51.9 % and Y=27.73+1.08[NO]; R^2=56.47. These results lead to the following conclusions: during the daytime, the linear correlation between NO_x and NO_2 is quite good, while for the nighttime the correlation between concentrations of NO_x and NO is very strong.

The variations of diurnal and nocturnal values of O_3 concentrations as a function of NO_x level are represented in Figure 12. The total value of O_x raised with NO_x, and the data have been linearly adjusted. Due to the influence of photochemical reactions on the O_3 formation, differences between values and linearly adjusted equations of day and night have been found. It is noticeable that the total local O_x has a contribution independent of NO_x and another one that is dependent. The first part is a regional contribution comparable to the regional level of O_3, while the last term is a local contribution effectively correlated to the level of primary pollution. It has been verified that the dependent local contribution of NO_x to O_x during the night is 37% lower than during the day.

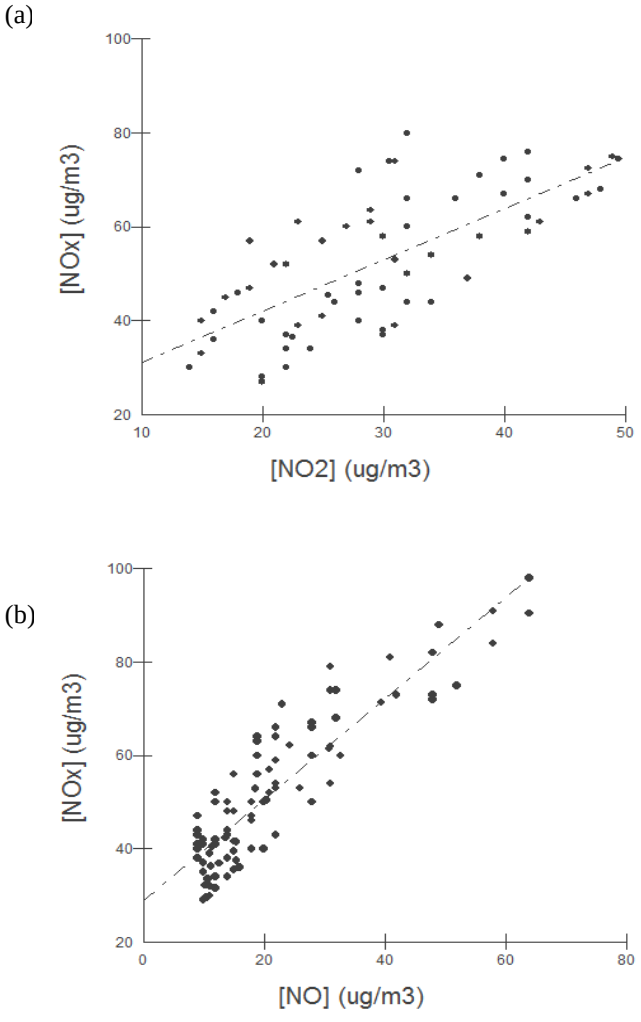


Figure 10. The average values of NO_x plotted as a function of (a) NO_2 and (b) NO for the daytime period.

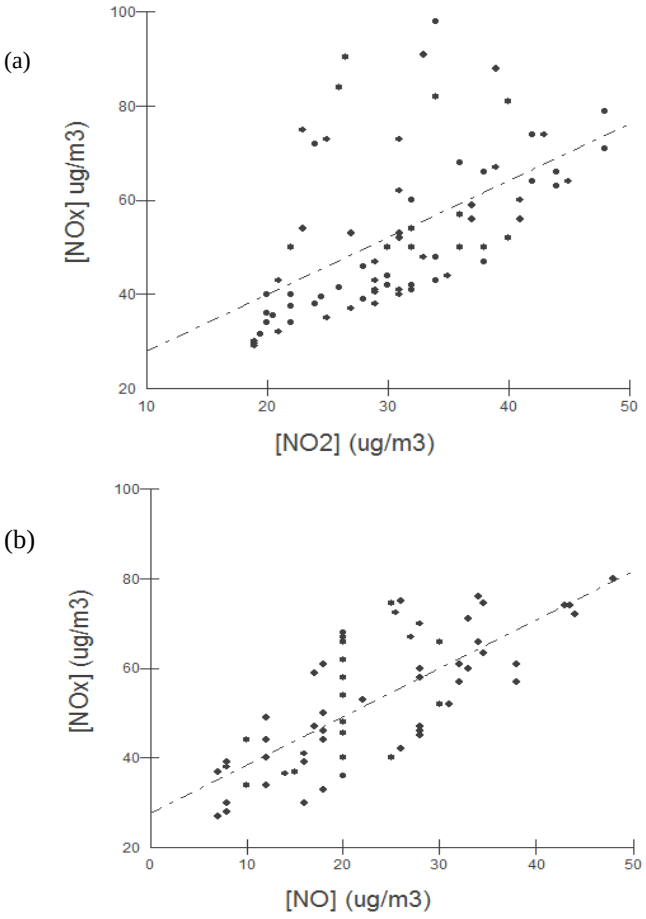


Figure 11. The average values of NOx plotted as a function of (a) NO2 and (b) NO for the nighttime period.

Local and regional contributions to the oxidant

However, the approximately 75 μg m^{-3} regional contribution is almost equivalent during the day and night. The regional contribution to O_3 is consistent with the values observed by. [5, 7] This result implies that the problem of air quality in Campo Grande is not just a local question, especially the street pollution, but also a regional issue of Campo Grande County. The territorial aspect observed for the analysis of O_3 pollution is consistent with the results reported by. [5, 24]

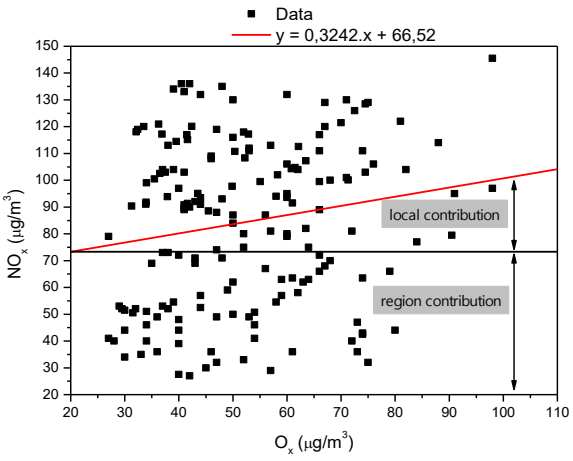


Figure 12. Variation of daily average NO_x concentration plotted as a function of O_x.

CONCLUSION

The present results indicate that the diurnal cycle of ozone concentration has a peak around noon and smaller nocturnal concentrations. The ozone concentration slowly increases after the sun rising, reaching a maximum value during the daytime and, in the sequence, decreases until the next morning. This is due to the photochemical formation of O_3. The shape and amplitude of ozone cycles are strongly influenced by meteorological conditions (solar radiation) and by the prevailing levels of precursors (NO_x). At the studied region, the daily cycle of NO concentration stem from vehicle emissions and its conversion to NO_2 has a huge impact on the daily cycle of ozone levels. A linear correlation between NO_2 and NO_x has also been determined, as well as between NO and NO_x, while a polynomial correlation between O_3 and NO_2/NO has been found. These forms can be useful for the strategies of O_3 provision and efforts of air pollution control. The O_x level is influenced by independent and dependent contributions of NO_2. The first one is due to the regional concentration of O_3, and the last one is correlated with the local level of primary pollution.

ACKNOWLEDGEMENTS

This work was supported by the Institute of Physics of the Federal University of Mato Grosso do Sul

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Received: 21.05.2017.

Accepted: 08.07.2017.

ISOLATION AND ANTIMICROBIAL ANALYSIS OF A STEROIDAL TERPENE FROM THE BUTANOL FRACTION OF *BYROPHYLLUM PINNATUM* (LAM.) OKEN

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Keywords: Butanol, chromatography, terpene, bacteriostatic, *Bryophyllum pinnatum*.

Bryophyllum pinnatum (Lam.) Oken is a plant used in treatment/management of ear-ache, cough, gastro-intestinal disorders and inflammation. Prior to this study, reports of the isolation of cardiac glycosides from the ethyl-acetate fraction of the plant abound. However, very scanty or no literature exists on other organic fractions from where chemical constituents could also be obtained. Hence, the chemical and biological properties of the butanol fraction of the plant were studied. The silica gel column chromatography of the fraction led to a steroidal terpene whose identity has been revealed to be 3-hydroxy-(3β, 17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one using the MS and IR spectral techniques. This compound was strongly bacteriostatic against *Staphylococcus aureus* and *Candida albican* sbut recorded no activity against *Escherichia coli*.

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Introduction

Bryophyllum pinnatum (Lam.) Oken syn, (*Cotyledon pinnatum*, *Crassula pinnatum* and *Kalanchoe pinnatum)* is known as miracle leaf or life plant grows as succulent perennial herb in the tropical climatic zones of Africa, Latin America and Asia. However, the plant is now cultivated on a large scale and sold to the pharmaceutical industry for economic benefits.[1] The plant is used in the treatment of ear-ache, cough, gastro-intestinal disorders and leucorrhoea. Furthermore, extracts of the plant are employed in the treatment /management of inflammations such as cardiac problems, wounds, sores, diabetes, liver problems, certain cancers and kidney troubles.[2] Previous chemical investigations of the plant have led to the isolation of three cardiac glycosides namely, bryophillia A, bersaldegenin -3-acetate and bryophillin C while the fractionation of ethyl-acetate marc yielded seven kaempferol rhamnosides.[3] The present study was carried out to isolate chemical constituent(s) in the butanol fraction which showed a higher antimicrobial activity than that by the ethyl-acetate fraction[4] and also screen the compound(s) for possible antibacterial and antifungal activities.

Experimental

Collection of plant material

The fresh leaves of *B. pinnatum* were collected in the month of July, 2016 from inside the University of Uyo Main Campus, Akwa Ibom State, Nigeria. The plant had previously been identified[4] and a voucher specimen of the plant (No. H 045) was deposited in the Herbarium Unit of the Faculty of Pharmacy. Immediately after collection, the leaves were dried in a laboratory oven at 40 ^{0}C for 48 h and the resultant material powdered on an electric mill (Uniscope, England).

Extraction and isolation

The dried powder (0.5 kg) was exhaustively extracted with 50 % EtOH (3 x 5 L) at room temperature (27±2 ^{0}C) for 72 h. The resultant crude extract mixture was filtered, concentrated *in vacuo* on a rotary evaporator, when 47 g of dried crude extract was obtained and stored in a desiccator prior to further use.

Subsequently, 7.8 g of the extract was partitioned using H_2O: BuOH (3 x 200 mL). The combined butanol fractions were evaporated to dryness to give a brown solid residue. Then 0.8 g of the fraction was chromatographed on a silica gel 254 column (Pyrex, USA; 8 g pre-swollen in 100 % toluene; 2 g concentration zone + 6 g separation zone; 10.2 x 4 cm) and eluted with a gradient of 20 % $(CH_3)_2CO$: toluene (36 mL), 40 % $(CH_3)_2CO$: toluene (36 mL), 60 % $(CH_3)_2CO$: toluene (36 mL) and 80 % $(CH_3)_2CO$: toluene (36 mL). Fractions of 6 mL each were collected and monitored on silica plates in $(CH_3)_2CO$:toluene:H_2O (10:20:1) using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents. Hence, fractions with similar TLC characteristics (R_f values, reaction with vanillin-H_2SO_4 spray) were bulked and dried. Three sub-fractions coded KF-1, KF-2 and KF-3 were obtained. Further TLC examinations of these sub-fractions in $(CH_3)_2CO$:toluene:H_2O (10:20:1) and $(CH_3)_2CO$:EtOAc (35:65) indicated a single spot in **KF-2**(yellow compound; R_f(0.57); 43 mg) while the others showed multi-component TLC profiles and were not processed any further in the course of this study.

Structural elucidation

The mass spectrum of the compound was run on Kratos MS 80 (Germany) while the IR analyses were done on Shimadzu FTIR 8400S (Japan).

Table 1. Antimicrobial screening of crude extract, butanol fraction, KF-2 at different concentrations on test microbes.

Test microbe	CE	BT	KF-2	DW	SP	NY
S. aureus (ATCC 21824)	11	16	18	5	27	5
E. coli (ATCC 23523)	5	5	5	5	31	5
C. albicans (NCYC 106)	10	12	15	5	5	28

CE= Crude ethanolic extract (20 mg mL^{-1}), BT = Butanol fraction (10 mg mL^{-1}), KF-2 (2 mg mL^{-1}), DW = Deionised water, SP = Streptomycin (10 μg mL^{-1}), NY = Nystatin (1 mg mL^{-1}).

Antimicrobial tests

The micro-organisms used in this study were limited to three *viz*: one Gram(+), one Gram(-) and a fungus. *Staphylococcus aureus* (ATCC 21824), *Escherichia coli* (ATCC 23523) and *Candida albicans* (NCYC 106) were clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fascitis, urine and wounds obtained from the Medical Laboratory, University of Uyo Health Centre, Uyo. The clinical isolates were collected in sterile bottles, identified and typed by convectional biochemical tests.[5,6] These clinical microbes were then refrigerated at -5 ^{0}C prior to use. The agar plates used were prepared by adhering to the manufacturer's instructions. The media and plates were sterilized in an autoclave at 121 °C for 15 min. The hole-in-plate agar diffusion method was used observing standard procedure with Nutrient Agar-CM003, Mueller-Hinton-CM037 (Biotech Limited, Ipswich, England) and Sabouraud Dextrose Agar (Biomark, India) for the bacteria and fungus respectively. The inoculum of each microorganism was introduced into each petri-dish (Pyrex, England). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Simax, India) to produce wells with diameter of approximately 5 millimeters. The wells were equidistant from each other and the edge of the plate.[7, 8] Concentrations of 20 mg mL^{-1} of crude extract, 10 mg mL^{-1} of butanol fraction, 2 mg mL^{-1} of KF-2 were introduced into the wells. Different concentrations of 10 μg mL^{-1} Streptomycin (Orange Drugs, Nigeria), 1 mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and deionized water were also introduced into separate wells as positive and negative controls respectively.[9-13] The experiments were carried out in triplicates. The plates were labelled on the underside and left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37±2 ^{0}C for 24 to 48 h. Zones of inhibition were measured in mm with the aid of a ruler.

Results and Discussions

Spectroscopic data

KF-2**:** $C_{22}H_{32}O_2$; yellow compound; R_f (0.57). MS [ES+-MS] *m/z* 328 $[M]^+$ (3.14 %), 285 $[M\text{-}2CH_3\text{-}OH+4]^+$ (5.25%), 273$[M\text{-}2CH_3\text{-}CO+3]^+$ (6.04%), 271 $[M\text{-}2CH_3\text{-}CO+1]^+$ (29.46%), 242 $[M\text{-}2CH_3\text{-}2CH_2\text{-}CO]^+$ (11.24 %) and 183 $[M\text{-}2CH_3\text{-}5CH_2\text{-}CO\text{-}OH]^+$ (100.00%). FTIR cm^{-1}: 913, 856 (alkyl substitution), 1612 (-C=C) and 3219 (-OH). The chemical structure of KF-2 was established by a combination of spectroscopic techniques as highlighted above. These data were matched with those in the library data of organic compounds. Furthermore, the obtained data were found to be consistent with those reported in literature.[14, 15] Therefore KF-2 has been identified to be 3-hydroxy-(3β,17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one. (Due to the nature of the matrix, many fragmented peaks appeared in the MS of the compound but those that are easily identifiable include $[M]^+$ at *m/z* 328 (3.14 %), while fragments at 285 (5.25 %), 273 (6.04 %) and 271(29.46 %) represent the losses of methyl groups and a hydroxy unit and methyl groups and carbonyl units from the molecular ion respectively. Furthermore, the ion a 242 (11.24 %), in addition to the excisions of methyl and carbonyl units also indicates the removal of methylene groups from the molecular matrix. However, the base peak at 183 (100.00 %) reveals the removal of methyl, carbonyl, methylene and hydroxy units from the $[M]^+$. The IR spectrum of KF-2 shows absorptions at 913, 856, 1612 and 3219 cm^{-1} indicating characteristic alkyl substitutions, endocyclic -C=C and -OH functional groups respectively. It is interesting to note that this steroidal terpene has been isolated from *Saccharium spontaneum* (L.) and *Rauwolfia vomitoria* (Afzel) using gas-chromatography/mass spectrophotometry.[14,15]

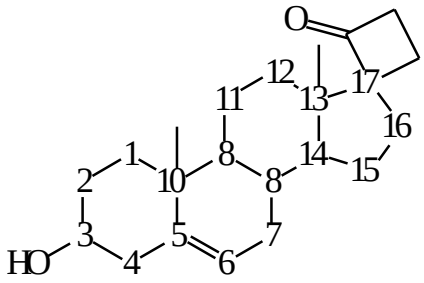

Figure 1. 3-Hydroxy-(3β,17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one

Antimicrobial Screening

The spectrum of microbes employed in the sensitivity tests was narrowed down to one Gram positive (*S. aureus*), Gram negative (*E. coli*) bacterial strains and fungus (*C. albicans*). The results displayed in the Table 1 show that the crude extract, butanol fraction and KF-2 are remarkably bacteriostatic against *S. aureus* and *C. albicans* while no activity was recorded against *E. coli*. Furthermore, the butanol fraction also inhibited the growth of *S. aureus* and *C. albicans* but was inactive against *E. coli*. Gram negative bacteria are well known for their unique resistance to antimicrobial agents. This resistance is believed to be due to the nature of the cell envelope of these organisms which unlike Gram positive organisms possess asophisticated

three-layered envelope which does not allow permeation of external agents.[16] Derivatization studies are currently on-going in our laboratories with the aim of improving on the observed activities.

Conclusion

The isolation of 3-hydroxy-(3β, 17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one is being reported for the first time from the butanol fraction of the plant. Hence, the compound is expected to serve as chemotaxonomic marker for this species and the genus, *Bryophyllum*in general. Furthermore, the results of the antimicrobial sensitivity tests lend some justification to the use of this plant especially in the treatment/management of bacterial disease. However, the compound and derivatives expected to be obtained in another study will be further screened against other bacterial and fungal strains with the aim of obtaining improved activity and widening the spectrum of antimicrobial activity.

Acknowledgements

We thank the Shimadzu Training Centre for Analytical Instruments (STC) Lagos, Nigeria for the assistance rendered in obtaining the spectra of the compounds. The expertise of E. Akpan, Principal Technologist, Pharmaceutical Microbiology Unit, University of Uyo, Nigeria in the conduct of the antimicrobial screening is warmly appreciated. The gifts of reagents by the Department of Pharmaceutical/Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria is heartily acknowledged.

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Received: 21.05.2017.
Accepted: 12.07.2017.

MALVA: FOOD, MEDICINE AND CHEMISTRY

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Keywords: *Malva*, *M. sylvestris*, antioxidant activity, ethnomedicine, flavonoids, medicinal uses.

Plants of the genus *Malva* (Malvaceae) have been used by humans for millennia. In addition to being an important nutritional source, they are used for many medicinal purposes. Modern research not only supports the ethnomedicinal uses of these plants but has discovered many others. Many review articles were published about the traditional uses of *Malva*, and some reviews were published about modern research findings of the medicinal and other properties of *M. sylvestris*. However, none of the review articles discussed both traditional and modern scientific knowledge about all plants of this genus. In the present article an attempt has been made to discuss both the aspects comprehensively. Conclusions have been drawn and future research possibilities suggested.

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Introduction

The genus *Malva* includes around 30 species.[1,2] The plants originally grew in Eurasia and North Africa, but humans have taken them to all continents except the two poles. Archeological findings in Jordan indicate a continuous use of *M. parviflora* by humans since ancient times.[3] Excavations in Isreal found an earlier use of *M. parviflora* and *M. aegyptiaca* dating around 23000 years before present.[4] These findings were confirmed and expanded by recent two studies of the same site.[5,6] Ancient Balkan inhabitants used *Malva* (unspecified sp.) as food sources and the materials of the plants were found in the remains of their teeth that go back at least 8600 years ago.[7]

The published literature about the *Malva* species is enormous but it focuses mainly on four of them: *M. neglecta*, *M. parviflora*, *M. sylvestris* and *M. verticillata*. The reported traditional uses and modern research findings of these species will be introduced in separate sections of this article, but they will be discussed jointly with the other species in the discussion section. Other species were little studied and for some others, there are no scientific publications at all. The last group includes *M. aethiopica*, *M. assurgentiflora*, *M. brasiliensis*, *M. campestris*, *M. canariensis*, *M. dendromorpha*, *M. hispanica*, *M. microcarpa*, *M. microphylla*, *M. pacifica*, *M. preissiana*, *M. pseudolavatera*, *M. qaiseri*, *M. stipulacea*, *M. subovata*, *M. transcaucasica*, *M. tournefortiana*, and *M. trifida*.

Many review articles were published about the traditional uses of *Malva* species and we will cite them here, but to the best of our knowledge, there are no published review articles that summarize the scientific literature about all *Malva* species that were studied so far.

M. verticillata was mentioned in a review article for its antidiabetic activity,[8] and wound healing potential of *M. neglecta*, *M. parviflora* and *M. sylvestris* was presented due to their anti-inflammatory properties.[9] Antitussive activity of *M. sylvestris* was reviewed among other medicinal herbs that have the similar activity.[10] A comprehensive review article about flavonoids in plants of the Malvaceae family (that includes the genus *Malva*) has been published recently and it shows some of the important natural products of this compound family.[11] In Figure 1, the structures of selected flavonoids are presented.

M. sylvestris is the most studied species of the Malva genus so far. An excellent review of traditional uses (food, ethnomedicine) and scientific research findings of this plant was published by J. Gasparetto et al. in 2011.[12] But it has three disadvantages. First, in terms of active natural products present in *M. sylvestris*, this review focuses only on polyphenols that are presented with structural formulae, while other compound families are mentioned but not presented like polyphenols. Second, a notable number of the cited references are patents that readers will not easily access. Third, despite being a very important research center of traditional uses and modern research of medicinal plants, Iranian publications are not sufficiently cited in this review.

Another review article that presents the biological activities of *M. sylvestris* was published by D. Paul.[13] It presents some traditional uses of the plant and most of its modern research findings of medicinal and other activities very briefly. The phytochemical part is very short and presents mainly polyphenols. The author presents seven major biological activities of the plant while ignoring many others. Finally, a review article that includes both traditional uses and scientific data about the medicinal properties of the plant was published by Shokrollahi and Ali.[14] Despite being supposed to include very much data, it presents it just partially and much of the known literature is not cited at all.

Traditional uses of *Malva* species

Three species possess the vast majority of publications, in terms of traditional-ethnobotanical uses viz., *M. neglecta*, *M. parviflora* and *M. sylvestris*. The details are presented in a separate tables (Tables 1-3), while the same about rest of the species are presented collectively in Table 4.

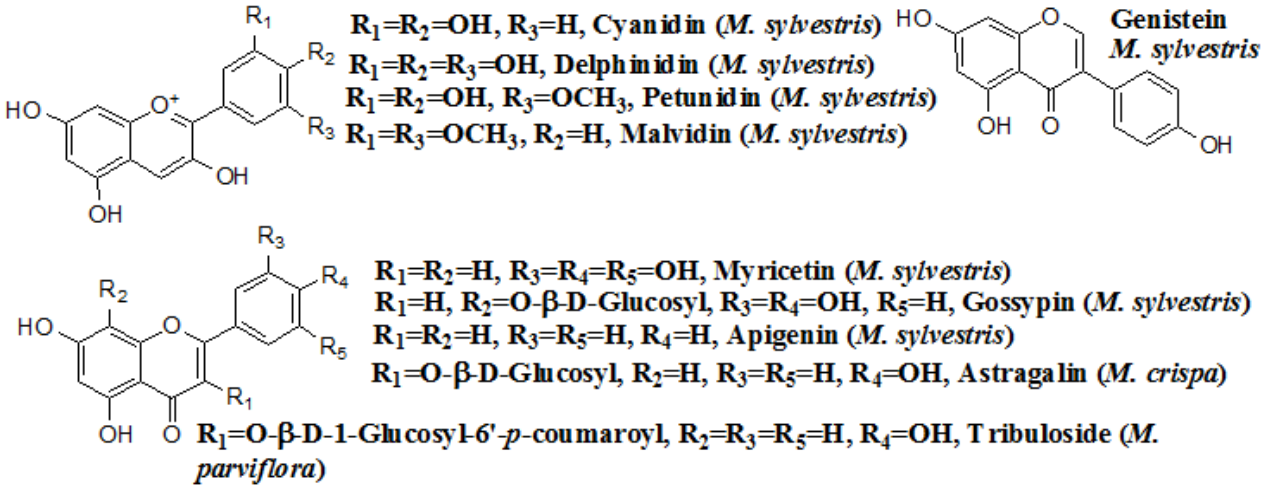


Figure 1. Selected flavonoids in three *Malva* species[11]

Table 1. Traditional uses of *M. neglecta*.

Country/Region	Used part/s	Use/s (reference)
Turkey/Germany	leaves	Compressed fresh leaves are used to treat abdominal pains[15]
Turkey	Stem, leaf, petiol	Eaten fresh or cooked[16]
Spain	Fruits	Immature, raw eaten as a snack[17]
Poland	Leaves, fruits, seeds	Leaves eaten in the past, immature, raw fruits still eaten, seeds grounded and added to bread[18]
Lebanon	Leaves	Infusion for treatment of gout and rheumatism[19]
Poland	Fruits	Immature, eaten raw [20]
Pakistan	Unspecified	Demulcent, aphrodisiac, laxative[21]
Pakistan	Shoots, roots	Shoots are used as potherb. The roots are used as purgative for young cattle[22]
Italy	Flowers, roots, leaves	To treat abdomenal pains. Leaves together with oil cure burns and zoster like inflammations. A hip-bath taken in the decoction soothes the uterus[23]
Turkey	Aerial parts	Decoction, to treat stomachache[24]
Pakistan	Roots	Purgative for young cattle[25]
India	Leaves	To heal broken bones and sooth baby's sore back.26 As anti diarrheal agent for calves and a food[28]
Turkey	Aerial parts	As food in various forms[27]
Iran	Aerial parts	Decoction to treat inflammation and sedative[29]
Pakistan	Roots, leaves, flowers	Roots used as purgative for young cattle. Leaves and flowers are used as demulcent, for bruise, inflammations, insect bites etc. Internally in the treatment of diseases of respiratory system digestive or urinary systems[30]
Pakistan	Whole plant	To treat piles and cough[31]
Iran	Flower, fruit	To treat sore throat and antitussive,[32,33] and as febrifuge[33]
Iran	Aerial parts	Decoction, poultice; laxative[34]
Spain	Aerial parts	Infusion to treat colds. Raw, immature fruits as food[35]
Pakistan	Stem	As food and to remove constipation and enhance digestion[36]
Pakistan	Leaves, roots	Leaves are used as food. Root extract is used to remove kidney stones[37]
Daghestan	Aerial parts	Food (cooked leaves, unripe fruits)[38]
Turkey	Aerial parts	Cooked or raw as salad[39]
Armenia	Aerial parts	Cooked or raw as salad[40]
Turkey	Whole plant	For large number of disorders[41]
Pakistan	Seeds	Crushed and used to cure cough and bladder ulcer[42]
Iran	Leaf, stem	Blood purification (unspecified method)[43]
Turkey	Leaves	To treat coughs and to treat abdominal pain[44]
Switzerland	Aerial parts	Ethnoveterinary: To treat skin afflications and orally to treat gastrointestinal disorders[45]

Table 2. Traditional uses of *M. parviflora*.

Country/Region	Used part/s	Use/s (reference)
Spain	Fruits, leaves	Immature, raw fruits as a snack, tender leaves and stems stewed[17]
Italy	Flowers, roots, whole plant	Flowers and roots are used against gastrointestinal pains as a laxative and as a diuretic. A decoction of the herb is used to treat gingivitis while cataplasms are used to treat furuncles and ulcerous wounds[23]
Turkey	Aerial parts	Food[27]
Pakistan	Leaves, roots, seeds	Seeds are used in cough and bladder ulcer. Leaf decoction is used for tap worm and profuse menstruation. Roots are used as sex tonic. Plant is also used as laxative[42]
Costa Rica	Whole plant	Ornamental plant[46]
Bolivia	Aerial parts	Anti-inflammatory, colds, fever, headache, vulnerary[47]
Canada	Aerial parts	Treat wounds of ruminants (unspecified method)[48]
South Africa	Unspecified	Poultice to treat sores and decoctions for neuralgia and sore throat[49]
South Africa	Leaves	To treat infected eye[50] and diarreea[51]
Pakistan	Seed, leaf	Seeds decoction is used as tea to treat common cold and cough. Leaves are cooked to treat constipation and used as vegetable[52]
Pakistan	Whole plant	Decoction used to cure cough flue and fever[53]
Iran	Leaves	Infusion, laxative, relieve cough and chest discomfort[54]
Argentina	Leaves	Decoction used in ethnoveterinary: intestinal colic, ocular diseases, wounds and injuries, mastitis and udders of goats and cows[55]
Bolivia	Leaves	Infusion, liver disorders, gastritis, stomach problems, renal inflammation, diuretic[56]
Iran	Seeds	Decoction to treat cold[57]
India	Whole plant	Decoction is used to treat cough, flu and fever[58]
Pakistan	Leaves	Food, decoction to treat constipation, cough and fever[59]
Egypt	Whole plant	Unspecified method, to treat pyorrhea, astringent[60]
Pakistan	Leaves, seeds	Infusion is used to lessen skin allergy, diaphoretic, headache[61]
Iran	Leaves, flowers	Food, treatment of kidney and bladder infections, emollient[62]
Pakistan	Leaves	Used by humans as vegetable, given to the hens as food[63]
Kashmir	Leaves	Eaten as vegetable, anthelmintic[64]
Chile	Unspecified	Antifungal[65]
Morocco	Leaves, stem	Decoction, respiratory disorders, cataplasm[66]
Kashmir	Shoot, leaf	Shoots are used for constipation, leaves are used to treat dry cough, bladder worm, diabetes and as vegetable and fodder[67]
Morocco	Leaves	Cataplasm: oral affection[68]
Pakistan	Seeds, leaves	Seeds used to relieve cough, bladder ulcer, leaves to treat tape worm, profuse menstruation[69]
Pakistan	Whole plant	Decoction, to treat cold, fever and cough[70]
Saudi Arabia	Leaf	Treatment of scorpion-sting[71]
Pakistan	Leaves, seeds	Leaves are emollient. Seeds are used to treat cough and ulcers in bladder[72]
Iraq	Leaves	Decoction: hair loss, abdominal pain, diarrhea[73]

Table 3. Traditional uses of *M. sylvestris*.

Country/Region	Used part/s	Use/s (reference)
Turkey/Germany	leaves	Compressed leaves are used to treat abdominal pains
Turkey	Stem, leaf,petiol	Eaten fresh (salad) or cooked[16]
Spain	Flowers, fruits, leaves	Flowers, as herbal tea and for making liqueur, immature fruits raw as a snack, tender leaves and stems stewed[17]
Poland	Leaves, fruits	Leaves used to be cooked in the past, immature, raw fruits eaten as a snack[18]
Lebanon	Leaves, flowers	Used to treat rheumatism and arthritis[19]
Poland	Fruits	Immature, eaten raw[20]
Pakistan	Unspecified	Medicinal, food for humans and animals[21]
Italy	Flowers, roots, leaves	To treat abdomenal pains, leaves are used to treat burns and zoster like inflammations, to soothe the uterus and as laxative and diuretic. It is used to treat furuncles and ulcerous wounds[23]
Turkey	Aerial parts	Used as food in many ways[27]
Iran	Flower, fruit	To treat febrifuge, respiratory ailments, depurative, mouth ulcers,[32] jaundicea and constipation[33]

Spain	Whole plant	Leaves and flowers are used for coughs, colds, sore throat, bronchitis, asthma and skin inflammations. Fflowers and leaves are used for fluid retention.[35]
Switzerland	Aerial parts	Used to treat skin afflications and gastrointestinal disorders in animals[45]
Costa Rica	Whole plant	Unspecified method: ornamental[46]
Argentina	Leaves	Used to treat intestinal colic, ocular diseases, wounds and injuries, and mastitis and udders of goats and cows[55]
Morocco	Leaves, roots	Decoction or cataplasm, respiratory or urinary disorders[66]
Arab countries	Leaves, roots, flowers	Many uses relates to *M. sylvestris* and *M. rotundifolia* together[74]
India	Leaves	Decoction mixed with lime juice to treat snake bite[75]
Italy	Different parts; Whole plant	Used to treat toothache, gingival inflammations, cough, cold, sore throat, mouth inflammations, diverse inflammations, furuncles, abscesses, wounds hemorrhoids, abdominal colic, warts, aphate, urinary infections and nail infections; also as perfume, analgesic, antitussive, anthelmintic, sedative and laxative.[76,79,80,82,85,88] To wash cows‘ udders;[83] in ethnoveterinary[88]
India	Unspecified	To treat tuberculosis[77]
Pakistan	Leaves	Unspecified Method: demulcent, aphrodisiac, laxative[78]
Cyprus	Leaves	Food, unspecified method, against cough and infection[84]
Spain	Aerial parts	Infusion: gastralgia, dysmenorrhoea, kidney malfunction, cold,[86] laxative, contusions, bruises, *Urtica diocia* stings and fever[87,91]
Portugal	Unspecified	Unspecified method: treatment of infections[89]
Syria	Leaves, flowers	Used as mouth wash, cough, respiratory infections, laxative[90]
Turkey	Aerial parts, whole plant	Wounds and furuncle,[92] hepatic and stomach disorders, cancer, sore throat, mumps, cough, cold, wounds, abscess, woman diseases, diabetes;[93] as food,[94] gynic problems, as green dye[102]
Slovakia	Aerial parts	Food[95]
Brazil	Unspecified	Infusion: cleanser, diuretic, boil, uterine inflammation, rheumatism, tonsillitis wound[96]
India	Aerial parts	Eaten twice a day to strengthen weak eye sight[97,112]
Algeria	Leaves	Unspecified method: laxative, hypoglymic[98]
Cyprus	Leaves	Cooked and consumed daily: antidiabetic[99]
Turkey	Leaves	Boiled and used to treat gastralgia, laxative[100]
Mexico	Flower, leaf	Capsules of extract (unspecified): weight loss, anti-inflammatory, laxative, antihyperglycemic[101]
Algeria	Aerial parts	Decoction: anti-inflammatory,weightloss[103]
India	Different parts of whole plant	Mucilaginous, cooling, anti-inflammatory, sore throat, jaundice, enlargement of spleen. cough, ulceration of urinary bladder, stimulates uterus, intestines[104]
Jordan	Leaves	Soaked (oral): emollient for intestinal mucosa[105]
Italy	Leaf, root, flower	Crushed leaves: teethache, whitlow. Leaves decoction or infusion: belly pain, cystitis, cough,cold, weightloss, bronchitis[106]
Iran	Flower	Infusion: jaundice, pharyngitis, furuncles, aphthous ulcers, antitussive[107]
Europe	Aerial parts	Abdominal colic, tympanism, rumination, diarrhea, constipation.[108]
Turkey	Roots	Infusion: abortive[109]
Algeria	Flower	Infusion: to treat abscesses, boils, swelling, insect bites, softening, antiseptic, astringent, abdominal pain, colic, otitis, asthma, constipation, colds, canker sores,[110] antiseptic for reproductive system[111]
Iran	Different parts	Immunomodulation, respiratory diseases of animals,[113] laxative, swellings, lubricant, clear the lung, expectorant, cough,[114] laxative, cough etc.[115]
Pakistan	Leaves	Unspecified method: bladder ulcer, diuretic, indigestion, anti-inflammatory, gastric mucus, relaxing activity[116]
Palestine	Leaves	Decoction: 10 g boiled in 100 mL of water, the affected is rubbed twice a day[117]

Table 4. Traditional uses of *Malva* species (excluding subspecies in Tables 1, 2 and 3).

Malva species	Country/Region	Use (reference)
M. cretica	Turkey	Food[27]
	Spain	Aerial parts, infusion: gastralgia[86]
M. moschata	Turkey	Food[27]
	Spain	Infusion to treat colds, raw, immature fruits as food[35]
M. nicaeensis	Turkey/Germany	Leaves: To treat abdominal pains[15]
	Spain	Immature, raw fruits eaten as a snack[17]
	Lebanon	Decoction of whole plant used to treat arthritis[19]

	Italy	Used against gastrointestinal pains, gingivitis, furuncles and wounds[23]
	Turkey	Food,[27] tosilitis, anpyretic, antitussive[41] Analgesic, ulcer, woman diseases, cough, digestive disorders, rheumatism, expectorant,[93] Urtica sp. Prickles,[94] abortive, cough, kidney disorders[109,119]
	Spain	Flowers, infusion: anticatarrhal[87]
	Israel	Whole plant: Cough, wounds and skin diseases[118]
M. pussila	Daghestan	Aerial parts, food (cooked leaves, unripe fruits)[38]
	Armenia	Cooked or raw as salad[40]
	Pakistan	Different parts are used to treat scurvy, piles, skin diseases, cough, bronchitis and inflammation of bladder[42]
	Slovakia	Food[95]
M. rotundifolia	Italy	Used for abdominal pains, burns, inflammations and soothing uterus[23]
	Arab countries	Many uses but this reference relates to *M. sylvestris* and *M. rotundifolia* together[74]
	India	Cough, inflammation, ulceration of urinary bladder, haemorrhoides, skin diseases, fever[104,120]
	Iran	Cancerous wounds,[121] oral sores[122]
M. verticillata	India	Cough, pectoral complaints, piles, ulcer, urine complaints, stmach ailments, swelling, kidney pain and food,[123,124,125]
	Ethiopia	External wounds, anthrax,[126] expulsion of placenta in cow,[127] fever,[128] vomiting, dysentery, neck tumor[129]
Unspecified	Spain	Leaves, infusion: anti-inflammatory, vulnerary (also after pig castration), antifurunculosa, stomachache, cholagogue, emollient, gingivitis, aphtae[130]
Unspecified	Canada/Trinidad	Aerial parts, unspecified method, respiratory (horses)[131]
Unspecified	Brazil	Leaves, infusion, soothing, headache, blood pressure[132]

Table 5. Modern research findings of some *Malva* species.

Malva species	Property	Major findings (reference)
M. aegyptiaca	Water soluble polysaccharides partial analysis	Water soluble were isolated and their monosaccharide units were identified: galactose, rhamnose, arabinose, mannose and glucuronic acid with the weight percentage of 56.86 %, 8.46 %, 9.04 %, 5.05 % and 20.57 %, respectively[133,134] (See note after ref. 133)
	Heavy metals accumulation	This plant accumulated Cd^{2+} more than other plants that were growing near phosphate treatment industry in Tinisia. Average concentration in the stems: 28.9 ppm[135]
	Chemical composition and antioxidant activity	Chemical composition was analyzed by GCMS for volatile compounds, and metal ions concentrations were determined. Total phenolic content was found and tested for antioxidant activity (strong, DPPH)[136]
	Chemical composition, antioxidant activity, bread additive	Chemical composition was determined focusing on flavonoids, triterpenoids and fatty acids. Antioxidant activity was determined by (DPPH, Fe^{3+} reduction). As bread additive, it was found to improve the quality and nutritional value[137]
M. crispa	High protein content	When cultivated protein content can reach up to 20 % (dried)[138]
	Flavonoids in flowers	Various glycosylated falvonoids were isolated from the flowers of the plant and characterized by chemical analysis and spectroscopic methods[139]
	Synthesis of gold nanoparticles	Leaves of the plants were used as reductant in the synthesis of AuNP's from HAuCl4. These AuNP's were tested against food borne bacterial pathogens[140]
	Synthesis of silver nanoparticles	Aqueous extract (reductant) of the leaves of the plant was used to prepare AgNP's. These were tested against various pathogen bacteria and found effective.[141] They were also highly toxic for zebra fish (Danio rerio)[142]
M. mohileviensis	Antiinflammatory and antioxidant	Ethanolic and aqueous extracts of seeds and aerial parts, as well as crude polysaccharides from cold and hot water extracts exhibited a significant anti-inflammatory and antioxidant (DPPH) activity[143]
M. moschata	Antibacterial	Hexane, dichloromethane and methonal extracts were prepared then the dry matter was suspended in DMSO. It was active against several types of bacteria[144]
	Plant pigments	This work focused on chlorophylls and carotenoids[145]
M. nicaeensis	Heavy metals accumlulator	Very good heavy metals accumulator from polluted soils, especially Zn and As. Promising bioremediation agent[146]
	Inhibition of pancreatic lipase	Dried aerial parts of the plant were extracted with methanol and the dry extract was suspended in DMSO and tested for PL inhibition. Strong activity[147]
	Inhibition of hormone sensitive lipase	Dried aerial parts of the plant were extracted with methanol and the dry extract was suspended in DMSO and tested for HSL inhibition. Strong activity[148]

M. pusilla	Polysaccharide isolation and activities	Water soluble polysaccharide was isolated and its monosaccharide units were determined (qualitatively). It was tested for anti-inflammatory activity (strong)[149]
M. rotundifolia	Composition	General composition was determined for macronutrients, fatty acids and some minerals. No alkaloids found[150]
	Antimicrobial	Ethanolic extract was dissolved in DMSO and tested for antimicrobial activity (very weak)[151]
M. sinensis	Cadmium accumulation	Contaminated soil from lead-zinc mines was treatred with this plant (also known as *M. cathayensis*) and found to be hyperaccumulator[152]

Modern Research Findings

The *Malva* species very extensively researched by modern techniques also. However, like in traditional uses of these plants, the vast majority of publications focus on four species of this genus: *M. neglecta*, *M. parviflora*, *M. sylvestris* and *M. verticillata*. Summary of research regarding each of these species are presented in a separate table. Like in traditional uses, *M. sylvestris* is most studied is this respect also.

Malva neglecta

Malva neglecta is a widespread edible plant that can be found in Eurasia and North Africa. It has many common names, sometimes in the same culture, and this might be misleading. Modern research findings approve its known traditional medicinal properties, and it is being studied more and more. A summary of these findings is shown in Table 6.

Malva parviflora

Malva parviflora is the second most widely studied *Malva* species after *M. sylvestris*. Despite the fact that its natural habitat overlaps with that of *M. sylvestris* and *M. nicaeensis*, it is relatively easy to distinguish *M. parviflora* from the other two: its leaves are more cycle shaped than of the other two species, and the plant itself is much lower than the two others. Modern research started studying *M. parviflora* only in the last four decades and the major findings are summarized in Table 7.

Malva sylvestris

Malva sylvestris is the most widely studied species of the *Malva* genus, and one of the most studied in the whole plant kingdom. In most parts of its natural habitat, it is very widespread, and most people mean it when they mention the words *Malva*, mallow or Khubbaiza in the Middle East. Modern reported literature about this plant is very large and published regularly. We have tried here to cite most important publications and summarized them in Table 8. We have cited together publications, published in the last twenty years (as of 2008), that report the same property. If notable differences are presented, the publications has been cited separately.

Malva verticillata

Among the four major *Malva* species listed in Tables 6-9, *M. verticillata* is the least investigated and modern research publications about it are fewer. In addition, traditional uses of this plant are also fewer than the other three. Despite this, a very comprehensive work has been carried out by studying the chemical composition and biological activities of seeds.

This exceptional work was done by a Japanese group lead by M. Tomoda and was published in nine articles between 1987 and 1992. This work has been presented collectively.

It is also notable that *M. verticillata* seeds are rich in polysaccharides that were thoroughly studied. It is interesting to pay special attention to the antidiabetic activity of *M. verticillata*, as this activity is one of the major properties of this plant.

Discussion

While studying the literature of traditional uses of Malva species, we found that some of them are in an ambiguous status as for their distinctiveness. For example, two of these species are M. alcea and M. excisa.[353] So, despite the fact that M. alcea was mentioned with other known Malva species (*Crispa*, *Pussila* and *sylvestris*) as having traditional antiviral infections activity of humans and animals,[354] we did not include this paper in Tables 3 and 4.

Al-Asmari et al. from Saudi Arabia reported that in ethnobotanical medicine of their country, leaves of *M. parviflora* are used to treat "scorpion sting envenomation."[71] They also cite another publication that is supposed to support their claim.[355] But reading this reference carefully reveals the fact that either the report or the citation or both are wrong: in reference 355 there is no mention of "Malva" and/or "parviflora", and authors indicate *Eryngium creticum* Lam. as a plant that is used in traditional Palestinian medicine to relieve pains of scorpion stings and snake bites.

Malva species are used for dye production.[102] The colored compounds of these plants, especially flavonoids/anthocyanins are the major source for these colors.

These compounds are naturally produced (see Figure 1), but this production can be enhanced by irradiation of the plants with UV,[285] or by the addition of other growth promoters. These results were confirmed by later publications.[287,288] J. Pinela and his colleagues from Portugal reported using Gamma radiation for the same purposes in M. neglecta.[356] Scientifically speaking, new results were obtained, and this report should be included in Table 6. We did not include it there for two reasons. One, this method is very expensive, and two, there are no follow up studies about the effect of this powerful radiation on other compounds in the plant, and maybe diverse results in terms of toxic materials production.

One of the very interesting medicinal activities of Malva plants is their potential use as antiobesity agents. In most publications, this activity is mentioned or studied along with the antidiabetic activity. *M. sylvestris* is traditionally used for this matter (weight loss) in Iran,[101,103] and this use is not "very unusual and rarely mentioned," as Menale and Muoio indicated.[106] Modern research found a possible explanation for this potential activity of *Malva* species. Y. Bustanji et al studied the antiobesity activity of *M. nicaeensis*, through the inhibition of pancreatic lipase (PL) and hormone sensitive lipase (HSL).[147,148] PL inhibition was also reported by *M. neglecta*.[171] These studies suggest the importance of followup studies on this subject.

Ameri et al indicated that the common name of *M. sylvestris* is "Jews mallow".[114] This is not correct because it refers to "Mulukhiyah" or *Corchorus olitorius*, a plant in the *Malvaceae* family but to the genus *Corchorus* not *Malva*.

Variations in chemical compositions of plants due to various factors such as locations, seasonality and weather conditions, are well known and widely reported. But such variations can also result not only from these external effects, but they can also result from internal factors of the plant e.g., the stage of growth or plant part. An interesting example of such variations has been reported by Zouari et al.[136]

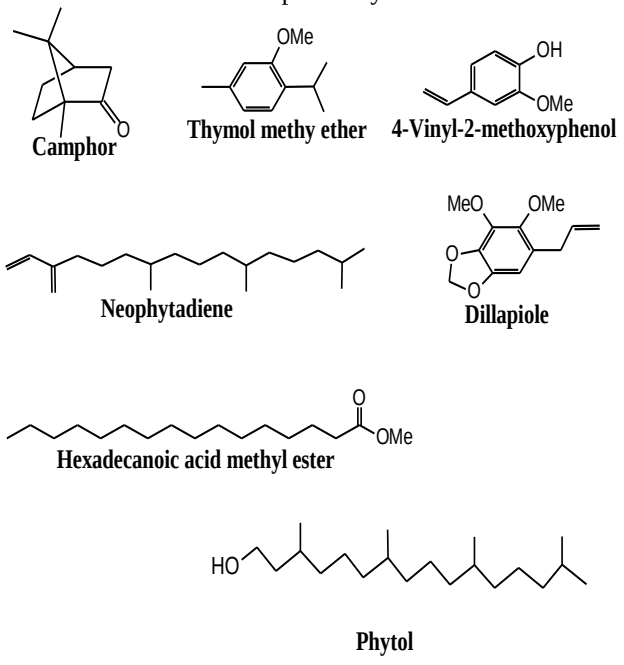


Figure 2. Compounds with notable concentration variations in *M. aegyptiaca* according to growth stage or plant part[136]

They have reported the different concentrations of volatile compounds according to three growth stages of *M. aegyptiaca* and also the concentration differences of macronutrients, metal ions and volatile compounds in leaves and fruits. Some of these compounds are presented in Figure 2, had notable variations according to growth stage.

Tunisian group lead by N. Zouari, published earlier this year another study[137] completing their previous report and evaluating the importance of *M. aegyptiaca* as bread additive. One of the interesting compounds they found was malvasterone (Figure 3), a steroidal triterpenoid that was first isolated from the roots of *M. parviflora*.[357] Steroidal units were also isolated later from *M. sylvest*ris (Figure 3).[286] These have an interesting lactone sub-unit, which grant them additional potential activities.

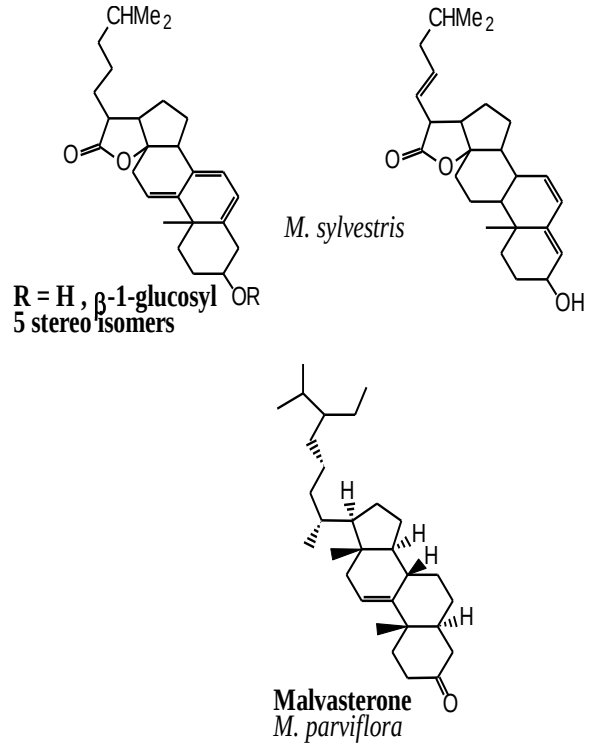


Figure 3. Structure of malvasterone.

Synthesis of nanoparticles of precious metals (or their oxides) using *Malva* species extracts has been reported by many groups.[140-142,172,188,216,303-304] In all these reports, aqueous extracts of leaves or flowers of the plants were used. The major quality that enables this kind of uses and reactions is the fact that these extracts are rich in flavonoids and anthocyanins. These compounds are good reductants, especially when hydroxyl (OH) groups are present in their structures.[358]

Beyrami-Miavagi and his colleagues,[164] have reported two properties of *M. neglecta*, antioxidant and contraceptive, as a safe alternative to prostodin (synthetic contraceptive), which causes oxidative stress and other damages in urinary system. There is no question about the antioxidants of Malva plants, but our intense search for research reports that support contraceptive activity of these plants was in vain, despite the fact that other plants with this activity are well known.[359,360,361]

The de-epoxidation of violaxanthin to zeaxanthin (Figure 4) is a biologically important process since it prevents photooxidative damages that might be caused to chloroplasts by excessive radiation.[362]

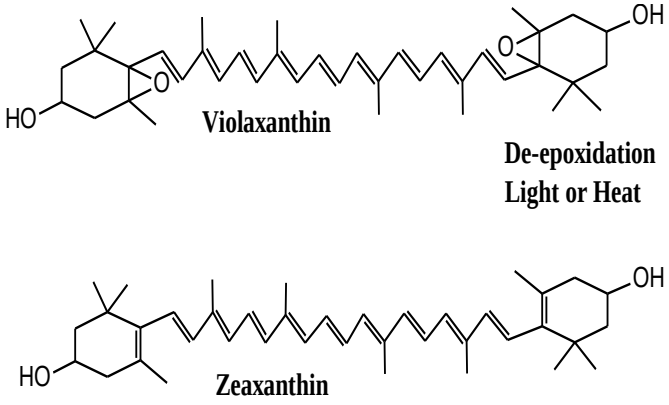


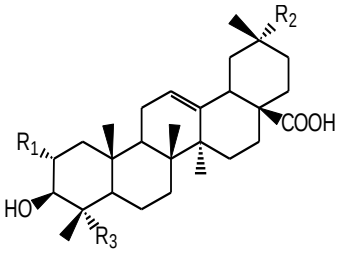
Figure 4. De-epoxidation of violaxanthin to zeaxanthin

This process occurring naturally has an additional importance when leaves of this plant are consumed as food since health benefits of zeaxanthin are more than those of violaxanthin. So it is favorable to expose leaves of the plant to light before cooking.

There seems to contradiction in two reports presented in Table 7 regarding the antidiabetic activity of *M. parviflora*. Phoboo[202] reported weak activity, however, strong activity has been reported by El-gizaewy.[204]

One can identify three sources of the difference. First the part of the plant used, leaves and seeds respectively; secondly, extraction solvent, water and n-hexane, and finally the testing model, in vitro enzyme inhibition (α-amylase and α-glucosidase) and in vivo treatment of streptozotocin (STZ) induced diabetic rats.

Isolation, characterization and three biological activities of a novel, natural derivative of the triterpenoid oleanolic acid has been reported.[210] Comparing the structures of oleanolic acid and the new compound (Figure 5), clearly shows that this derivative includes more polar functional groups.



Oleanolic acid: R_1=H, R_2=R_3=CH_3

Derivative (ref. 210): R_1=OH, R_2=R_3=CH_2OH

Figure 5. Oleanolic acid and its novel, natural derivative reported in reference 210

Oleanolic acid itself is physiologically very active compound,[363] and this new derivative should also be additionally studied and chemically modified to prepare new derivatives and test their activities.

Toxicity of Malva species to animals is very rare but was indicated in the research literature. *M. neglecta* was reported to cause hypocalcemia in cow,[205] but the mechanism of poisoning action was not reported. A controlled study with horses showed that consumption of large amounts of *M. parviflora* resulted in energy balance damages.[213] It is proposed that cyclopropene fatty acids contained in the plant are responsible for this, and elevation of acylcarnitine (Figure 6).

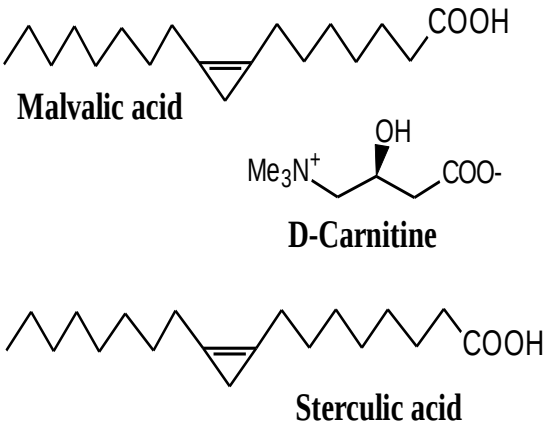


Figure 6. Structures of malvalic and sterculic acids and carnitine

Authors explain their findings by the rapid and easy oxidation of the unstable cyclopropene ring, which is consistent with earlier reports.[364]

In 2006, Ganai et al reported a detailed method of isolation and purification of the enzyme sulfite oxidase from leaves of *M. sylvestris*.[228] This work completes an earlier work by this author (and others), but we did not cite it in Table 8 due to its partial findings.[365] Sulfite oxidase catalysis the oxidation of sulfite to sulfate and it has very important role in sulfite detoxification in plants.[366]

Germination inhibition of plants by other plants is known as allelopathy. Many plants act as allelopathic for another plant and their growth can be inhibited by other plants. Cutillo and her colleagues found that two new terpenoids that they isolated from *M. sylvestris* and characterized have an allelopathic effect on *Lactuca sativa*.[229] The structures of these compounds are shown in Figure 7. Such findings are consistent with earlier and later reports of allelopathic effect of terpenoids on *L. sativa*.[367]

Wound healing and treatment of skin diseases by plant extracts and other pharmaceutics is a known phenomenon and has been extensively studied and published.[368] Plants of the *Malva* genus are among the most active as regard this activity. They were used for such purpose in many cultures.[35,42,45,61,76,81-83,104,118]

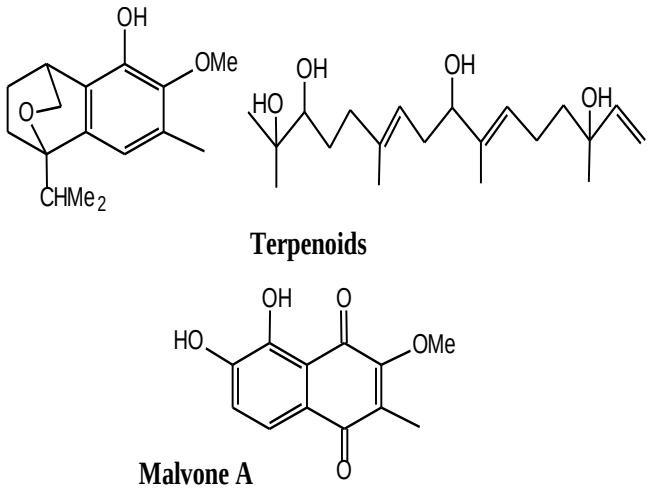


Figure 7. New terpenoids isolated from *M. sylvestris* (ref. 229) and Malvone A (ref. 230).

Modern research findings confirm the traditional knowledge.[161,183,248-258] But carefully reviewing this data reveals an interesting and not always consistent results. For example, it is reported that chloroform and n-hexane extracts of *M. parviflora* are irritant.[183] However, others found counter-irritant activity in chloroform and other extracts of the same plant.[369] Moreover, chloroform extract of M. sylvestris was also reported as having wound healing activity.[249,250] These contradictions must be resolved by future more extended studies.

Mineral content of *Malva* species has been published in several research articles. There is a common result in all of them. The genus Malva is rich with potassium and calcium (*M. neglecta*;[150] *M. parviflora*;[201] *M. sylvestris*[223,229,260,262,263]). Several methods were used to determine the metal content and one of the best, yet simplest was reported by S. Terfi and his colleagues.[264] Related to that, some studies were conducted to test the capacity of *Malva* species to accumulate heavy/toxic metals from contaminated soil or aqueous solutions. We present the results of these publications in Table 10.

Table 6. Modern research findings of *Malva neglecta.*

Property	Methods and major findings (reference)
Chemical composition	Alkaloids, flavonoids, saponins are present but tannins are absent.[153]
Antioxidant activity	Moderate activity was detected in methanolic and aqueous extracts DPPH and TBA methods.[154]
Poisoning	Hypocalcemia reported in a cow which ate a large amount of the plant.[155]
Antibacterial	Ethanolic extract of flowers was tested against 10 types of bacteria. Six of them were resistant[156]
Antibacterial	Aqueous, ethanolic, chloroform and acetone extracts show moderate activity against four types of bacteria.[157]
Antimicrobial	Aqueous, methanolic, n-hexane, chloroform, ethyl acetate and n-butanol extracts did not show any activity against four types of bacteria.[158]
Antioxidant	Stong antioxidant capacity of the phenolic content.[159]
Antioxidant	Hydroalcoholic (1:3) extract showed strong activity[160]
Antibacterial	Aqueous, ethanolic and chloroform extracts of M. neglecta and M. sylvestris were tested against seven types of bacteria that cause wound infections. All extracts were active, and ethanolic was most active for both plants. Extract of M. sylvestris was more efficient[161]
Mineral content	K^+ was highest with 230 mg kg^{-1} [162]
Antiinflammatory	Methanol extracts show moderate activity against some types of inflammation.[163]
Contraceptive	Female rats were treated with hydroacoholic (1:8) extract and prostodin, a synthetic contraceptive. The extract caused lesss oxidative stress and other damages in kidneys and urinary space[164]
Kidney stones preventive	Production of kidney stones (CaOx) and tubulointerstitial damage, induced in male rats by ethylene glycol and ammonium chloride, was reduced on treatment with aqueous extract of the plant.[165]
Antiinflammatory	Aqueous extract was tested against various inflammatory agents in patients with osteoarthritis and found as potent inhibitor[166]
Antibacterial	Ethanolic extract found active against antibiotic-resistant S. aureus[167]
Mucilage uses	Mucilage was separated from fresh leaves and precipitated by acetone, contains carohydrates and flavonoids. It was used to bind the synthetic analgesic of diclofenac sodium and produce granules[168]
Antioxidant	Hydrocolloid water extract of plant leaves was prepared in various temperatures (55-95 °C). The products of higher temperatures showed excellent antioxidant activity (DPPH)[169]
General composition, antioxidant, antibaterial	General composition of fresh leaves was determined by three classes of components: macronutients, metals and total phenols. The methanolic extract was tested for antioxidant and antibacterial activities: both were found moderate comparing with other plants in this study[170]
Health benefits, antioxidant and enzymes inhibition	This study connects the traditional known health benefits of the plant (very good food photos) and its medicinal properties. Aqueous extract showed antioxidant activity and pancreatic lipase and α-glucosidase inhibition[171]
Synthesis of silver nanoparticles	Aqueous extract was used as a reductant in the synthesis of AgNP's from silver nitrate[172]
Composition, anticholinesterase, antimicrobial, antioxidant, aflatoxin content	The aqueous extract was analyzed by LC-MS/MS for total phenolic content and determination of single phenolic compounds. It showed anticholinesterase, antimicrobial and antioxidant activities, and no aflatoxin content. The essential oil was analyzed by GC-MS for fatty acids content and volatile compounds[173]

Table 7. Modern research findings of *Malva parviflora.*

Property	Methods and major findings (reference)
Thiamine content and safety to ruminants	Thiamine content of the plant is relatively high compared with other plants in this study. This is in accordance with very low levels of thiamine destroying enzyme, thiaminase, that consumption of large amounts of it may lead to cerebrocortical necrosis in ruminants.[174]
De-epoxidation of in leaves	Temperature and light (λ=505 nm) on the de-epoxdation of violaxanthing to zeaxanthin. Leaves were sensitive to radiation and less to temperature, especially under 24 °C.[175]
Antifungal proteins	Two antifungal proteins were isolated and characterized from seeds of the plant. The proteins were found fungisatic instead of fungicidal.[176]
AChE inhibition	Ethanolic extracts of leaves are better inhibitor of acetylcholine esterase (AChE) than aqueous extracts and most efficient concentration was 0.025 mg ml^{-1}.[177]
Antibacterial, antiinflammatory	Extracts of plant parts showed antibacterial and anti-inflammatory activities. Authors suggested synergism of two unidentified anti-inflammatory compounds.[178]
Proximate composition, antioxidant	Protein, total phenolic content and metal content was determined. The plant is calcium rich. 80% Aqueous methanol extract showed moderate antioxidant activity (DPPH).[179]
Total phenolics, antioxidant	Total phenolic content was detemined and two of their sub-classes. Ruduction power (Ferric ion) and DPPH, ABTS quenching capacity were also determined[180]
Anti-inflammatory, anti-allergic	Aqueous extract of the flowers found active againts allergen-induced eosinophilia[181]
Anti-inflammatory, analgesic	Methanol extract of aerial parts has anti-inflammatory and analgesic activities.[182]
Antibacterial, antifungal, irritant	Hexane, chloroform, ethanol and aqueous extracts showed antibacterial and antifungal activities. Most extracts had irritant effect on inner surface of ear of male albino rabbits.[183]
Anti-inflammatory, antioxidant, metal chelating	Methanolic extract had stronger anti-inflammatory activity. Methanolic and aqueous extracts showed strong antioxidant (DPPH) and ferrous ion chelating activities.[184]
Phytochemical screening, antioxidant	Fresh leaves and stems were found to contain phenols, flavonoids, sponins, alkaloids, resins and tannins. Aqueous extract of both parts had antioxidant activity.[185]
Antioxidant	Ethanolic extract of leaves had strong antioxidant activity.[186]
Hypoglycemic	Extract of aerial parts showed promising antidiabetic activity.[187]
Silver nanoparticles preparation	Fresh leaves were extracted with 70% ethanol/water and the extract was used as reductant in synthesis of AgNP'S from $AgNO_3$.[188]
Phytochemical analysis	Seven phytosterol, two polyphenols and 14 fatty acids were identified from 85% ethanol extract. Alcoholic extract had significant anti-inflammatory and cytotoxic activities and aqueous extract antimicrobial activity.[189]
Phytochemical analysis	Very partial phytochemical analysis of aerial parts that were extracted with chloroform.[190]
Toxicity to Flour beetle	Ethanolic (see remarks in the discussion section) extract of leaves was prepared and found fatal for the larvae of *Tribolium confusum*[191]
Growth promoter	Aqueous extract (plant part/s not indicated) promoted the growth of cowpea (*Vigna Unguiculata*)[192]
Cytotoxicity	95% Ethanolic extract showed weak activity against breast cancer cell lines.[193]
Hepatoprotective	Whole plant was extracted by 70% ethanol/water. The extract was analyzed for major chemical constituents and tested against paracetamol induced hepatotoxicity: very active[194]
Neuroprotective	Leaves ethanolic extract is active against amyloid-β- (Aβ-) mediated alzheimer's disease.[195]
Pharmacognostic variables	Pharmacognostic variables were determined for all parts of the plant in order to standardize herbal medicines data.[196]
Heavy metals accumulation	Heavy metals concentrations was determined in aerial parts of the plant grown in normal soil. Results showed that heavy metals did not exceed healthy limits.[197,198]
Oil contamination removal	Soil was contaminated with oil (0.1 and 0.5%) and was treated by the plant for oil removal. After 30 days, 88.5% of the oil was removed.[199]
Adsorption of Cr(III)	Conatminated water with Cr(III) was treated by inserting the plan's roots in and the concentration change was measured under various pH and temprature values. Moderate activity[200]
Nutrients, antioxidant	Major nutrients, minerals, total phenolic content and anthocyanins were determined in aerial parts. High ntioxidant (DPPH) activity[201]
Antidiabetic, antioxidant, antihypertensive	Watet extract of aerial parts was prepared and its major chemical constituents were determined. It was tested for antidiabetic (weak), antioxidant (moderate) and antihypertensive (very weak) activities[202]
Drying process effect on content	Leaves were dried by four methods and total phenolic content and antioxidant activity of the products were determined[203]
Fatty acids content, antioxidant, antidiabetic	Dried seeds were extracted by *n*-Hexane to prepare fixed oil. The fatty acid content of this oil (9, saturated and unsaturated) and its antidiabetic (high) and antioxidant (high) activities were determined[204]
Antiprotozoal, antimicrobial	75% Ethnol/water extract of the leaves was tested for antiprotozoal and antimicrobial activities. It showed moderate antimalarial and high anti-leishmanial and weak antimicrobial activities[205,206]
Ulcerative colitis inhibition	Methanolic and aqueous extracts were prepared from leaves. Methanolic extract was more efficient in attenuating inflammation and tissue damage induced acetic acid[207]

Growth inhibition by eucalyptus leaf extract	Eucalyptus leaves aqueous extract was found relatively effective but only in high concentrations as herbicide against seeds (germination control) and adult plants of *M. parviflora*.[208]
Antioxidant	This study combines ethnobotanical uses (Brazil) and antioxidant (3 methods) test of the aqueous extract of leaves: high[209]
Hypolipidemic, hypoglycemic, anti-inflammatory,	A novel triterpenoidic acid, 2α,3β,23α,29α tetrahydroxyolean-12(13)-en-28-oic acid was isolated and was found to have hypolipidemic, hypoglycemic and anti-inflammatory activities.[210]
Memory retention	80% Ethanol/water extract showed memory retention in mice.[211]
β‑sitosterol, antibacterial	Chloroform and ethanolic extracts of roots showed high antibacterial activity against small number of bacteria. It is suggested that β-sitosterol in chloroform extract is the active compound[212]
Horse poisoning	Four horses, fed with large amounts of the plant, had myocardial disease and myopathy compared to control. Cyclopropene fatty acids in the plant may cause negative energy balance and abnormal acyl carnitine profiles.[213]
Zinc accumulation	High concentrations of Zn were found in roots but not aerial parts of the plant that grew around steel industries. [214]
Cd, Cu accumulation	Plants irrigated with sewage water, accumulated copper and cadmium up to six folds compared with control.[215]
Gold nanoparticles	70% Ethanol/water extract of the plant was used as reductant in the synthesis of AuNP's from $HAuCl_4$ [216]

Table 8. Modern research findings of *Malva sylvestris*.

Property	Methods and major findings (reference)
Antibacterial	Hexane, dichloromethane and methonal extracts were prepared then the dry matter was suspended in DMSO. Its antibacteria activity was very weak[144]
Plant pigments	Chlorophylls and carotenoids were isolated and identified[145]
Antibacterial	Aqueous, ethanolic and chloroform extracts of *M. neglecta* and *M. sylvestris* were tested against seven types of bacteria that cause wound infections. All extracts were active, and ethanolic was most active for both plants. Extract of *M. sylvestris* was more efficient[161]
Flavonoid glucoronides	Glucoronides (glucosyl derivatives) of various flavonoids from leaves of the plant, including a glucoronide sulphate[217,218]
Anti-complementary mucilage	Water extraction of fresh leaves afforded mucilage with average molecular weight of $6x10^6$. It consists L-rhamnose, D-galactose, D-galacturonic acid and D-glucurnic acid, with molar ratio of 6:3:2:2, respectively. This mucilage has anti-complementary activity[219]
Anti-complementary polysaccharide	Water extraction of fresh leaves yielded a polysaccharide with molecular weight of $1.1x10^4$. It consists L-rhamnose, D-galactose, D-galacturonic acid and D-glucurnic acid, with molar ratio of 22:6:22:11, respectively. This mucilage has anti-complementary activity[220]
Scopoletin isolation	Scopoletin (7-Hydroxy-6-methoxycoumarine) was first isolated from the plant by aqueous extraction (yield is higher than methanolic)[221]
Acidic polysaccharides	High molecular ($1.3\text{-}1.6x10^6$) acidic polysaccharides (mucilage) were isolated by water extraction. No activity reported[222]
Mineral content	Mineral content was determined from plant ash by means of atomic absorption. Among studied seven edible plants, *M. sylvestris* had the highest content of phosphorus and potassium[223]
Radical scavinging, Fe(II) chelating	50 % Methanol/water extract was tested for antioxidant activity (DPPH, H_2O_2) using low concentrations of the extract. For DPPH test it was inactive and for H_2O_2 it had moderate activity. Fe(II) chelating high[224]
Anthocyanin inhibibs lipid oxidation	"Homemade" anthocyanin samples from the plant were tested for radical scavinging in high fat albino rats. In concentration of 0.2 mg mL^{-1} it showed very strong activity of plasma lipid oxidation inhibition[225]
Anthocyanin antibacterial activity	"Homemade" anthocyanin samples from the plant were tested against several types of bacteria, and found active against some of them[226]
AChE inhibition, antioxidant	Ethanolic extract was prepared from aerial parts and tested for acetyl choline esterase inhibition: active only in high concentrations. Antioxidant activity was high[227]
Sulfite oxidase purification and kinetics	Sulfite oxidase was purified from leaves by acetone fractionation, heat treatment and several analytical methods. The kinetics, optimal pH, optimal tempretaure and the activation energy of the ezyme activity were detemined[228]
Novel terpenoids, growth inhibition of *Lactuca sativa*	Novel terpenoids (Figure 7) and other known were isolated from the aqueous extract. The new compounds were tested for germination inhibition of *L. sativa* and found moderately active[229]

Malvone A, antifungal	New phenolic 1,4-quinone (malvone A, Figure 7) was isolated from the stem bark by aqueous-acetone solution that contains ascorbid acid. The synthesis of malvone A in the plant was enhanced by plant pathogen *Verticillium dahliae* and it has antifungal activity against the same fungus[230]
Alkaloid content	Total alkaloids content was isolated from methanolic extract that was treated by acid then base: 35 mg in 100 g plant aerial parts[231]
Antiproliferative (inactive)	70 % Ethanolic/water extract of leaves was tester for antiproliferative activity against four types human tumor cell lines: inactive. *n*-hexane fractionation yielded campesterol, stigmasterol and γ-sitosterol[232]
Effect of aqueous sulfur dioxide	Treating the plant leaves with various concentrations of aqueous sulfur dioxide solution resulted in decrease of all biochemical activities especially antioxidant capacity[233]
Immunomodulatory	Carbohydrate rich water extract was tested for immunomodulatory activity in BALB/c mice and found active in some test types[234]
Anti-inflammatory, antioxidant	70 % Ethanolic extract was prepared and fractionated with 90 % CH_3OH and *n*-hexane. Tests showed antioxidant (DPPH) and anti-inflammatory (croton oil-induced ear oedema in mice) activities[235]
Anti-inflammatory	In the tests of cream prepared from water extract of aerial parts[236] and 70 % ethanol extract of flowers[237] against carragenin-induced edema in rats, and in tests of ethanolic extract of leaves against 12-O-tetradecanoylphorbol-acetate induced in mice active compounds were indicated. Quantification of prostaglandin by LC-MS/MS validated the tests of ethanolic extract of leaves was tested against activity of various inflammatory agents.[239] Different fraction of ethanolic extracts of leaves were tested against *A. actinomycetemcomitans.*[240]
Antinociceptive	Leaves water extract was not found active by hot-plate model against four pain agents, writhing test, neurogenic and inflammatory phases of formalin model, capsaicin-induced pain.[241]
Anti-inflammatory, anti-ulcerative	Aqueous extract of aerial parts showed anti-inflammatory and anti-ulcerative activities in rat model (drinking water)[242]
Antioxidant	Aqueous extract of leaves was tested for antioxidant activity (DPPH, superoxide radical), then analyzed to yield eleven active compounds, that each one of them was tested.[243] Total phenolic content and antioxidant activity (three methods: FRAP, DPPH, TAC-Mo^{VI}) of leaves (highest), stems and seeds.[244] Total phenolic content and antioxidant (DPPH) activity were determined for methanolic extract of whole plant.[245] Methanolic extract of leaves was teasted for antioxidant activity (DPPH).[246] Methanolic (with ammonium citrate) of aerial parts was teasted for antioxidant activity (DPPH)[247]
Skin disorders and wound healing	Burn wounds in rats were reated with diethyl ether extract of flowers, resulting notable healing.[248] Excision wounds were treated with chloroform extract of flowers, resulting wounds healing.[249,250] Fifty patients with hand eczema treated with ointment showed partial healing and no adverse effects.[251] Alloxan-induced diabetic rats with blade injury[252] and burn injury[253] showed complete and 80 % healing on treatment with dietheyl ether extract of flowers for 18 days. Blade-induced injury in palatal mucosa of rats showed on healing effect of 70% ethanol/water extract of stems and leaves.[254] 70% Ethanol/water extract of flower was prepared as cream with 5 and 10%. It was tested for treatment of burn (hot plate) injuries. Complete healing after 35 days.[255] Polyherbal cream (PHC) that contains aqueous extract of leaves was used to treat second-degree burn wounds in rats (110°C, 10 s). After 14 days, 87% of the wounds healed. Total phenolic content, antimicrobial and antioxidant activities PHC were determined.[256] A cream (1%) was prepared from aqueous extract of flowers used to treat surgical blade induced wounds in mice. After 10 days healing was almost complete.[257] 40% Ethanol/water leaves extract was used to treat inflammation (formalin induced) and tested for linear incised wound healing in rats and found effective[258]
Metal ion composition and heavy metal accumulation	Calcium and potassium were found in relatively high concentrations in leaves.[259] Unspecified plants showed high concentrations of calcium and potassium after burning.[260] Accumulation of major elements and heavy metals in plants (aerial parts) grew in industrial contaminated soil. The plant was classified as hyperaccumulator.[261] Concentrations of metals in leaves were measured in populated areas. Results are around standard except lead which was higher.[262] Metal composition of edible parts was determined by inductively coupled plasma optical emission spectroscopy. Results showed that washing the plants as done before cooking, reduces the content of metals notably.[263] This study reports three methods of extracting toxic metals from the plant leaves, finding that dry ashing method using 4:1 HNO_3:HCl was most efficient.[264] Dried flowers were sieved in different meshes and used as green biosorbent of Pb(II) from aqueous solution. Maximum biosorption capacity was 25.64 mg/g.[265] Dried leaves were powdered and dried again and the resulting mesh was used to Hg(II) from aqueous solutions comparing with charcoal and found more efficient.[266] Synergism of plant powder and charcoal yielded high removal of Hg(II) from aqueous solutions[267]
Antimicrobial, antibacterial, antifungal	Whole plant extract (96% EtOH/Water, traditionally used to treat gastrointestinal disorders) found active against *Helicobacter pylori*.[268] Methanolic extract of aerial parts and three fotmulations of it were tested against several bacteria. The extract was very active gainst *S. aureus*.[269] *n*-Hexane, dichloromethane and methanolic extracts of leaves and flowers were prepared (separately) and tested

	for antimicrobial and and antifungal activities. Only methanolic extract was active.[270] Tincture (no preparation method) was most active among other plants, against *Candida albicans* and *C. tropicalis*.[271] This study tested the components antimicrobial activity of commercial mouthwash Malvatricin: tyrothricin, hydroxyquinoline and the plant tincture. It revealed that activity is only a result of hydroxyquinoline presence.[272] Aerial parts were extracted with 50% methanol water and the extract was tested for urease inhibition: weak.[273] Methanolic extract of leaves was tested for urease inhibition (moderate) and against *Helicobacter pylori*: inactive.[274] Aqueous, ethanolic and *n*-hexane were prepared and tested against oral bacteria: inactive.[275] Roots were extracted with 48% EtOH/water and tested against oral *Streptococci* bacteria and found active against some of them.[276] Acetone/water (7:3) extract was tested for antifungal activity (moderate) and analyzed for major compound families and some single active compound.[277] Ethanol/water (7:3) extract of aerial parts was tested for antibacterial activity: active against *Pasteurella multosida*, inactive against *Salmonella enteritidis*.[278] Aerial parts aqueous extract was tested against *Candida albicans* infection in mice and found moderately active.[279] Vaginal protection medical products based on extracts of the plant (with extract of *Calendula officinalis*) are used, especially in pre-pubertal age.[280] Aerial parts 96% ethanol/water extract was tested against *Candida albicans* infection in mice and found moderately active.[281] Leaves were extracted with 3:1 methanol:water and the extract was tested against four types of bacteria: inactive.[282] Aqueous and ethanolic extracts of all parts were prepared and tested against *C. albicans* biofilm formation. All extracts were active but most active was the ethanolic extract of roots[283]
Chemical composition and some related properties	Methanolic extract of aerial parts was tested with four methods for antioxidant activity. Chemical composition was determined on three levels: macrontrients, major compound families and major compounds in each family.[284] Anthocyanin production was increased by UV irradiation of the flowering plant.[285] Three steroidal lactones and three glycosyl derivatives were isolated and characterized from methanoic extract of the friuts (see Figure 3).[286] Silver nitrate, abscisic acid and UV-B radiation used as growth-stressers and had different effects on growth of the palnt, but they all increased anthocyanin production.[287] Same findings of previous publication concerning silver nitrate.[288] Leaves and petioles methanol extract was analyzed (GCMS) for proximate composition: fatty acids, metal ions, total flavonoids and mucilage. The antioxidant capacityof the extract was tested by four methods.[289] Lipid content of seed oil (extracted with petroleum ether) was determined by GCMS for saponifiables (fatty acids by preparation of their methy esters) and unsaponifiables (mainly phytosterols). The antioxidant of the oil was determined by DPPH assay.[290] Ethanolic extract (plant parts not indicated) was tested fot antioxidant activity (DPPH) and partitioned with other solvents. Total content of the following compound families was determined: free monosaccharides (none), saponins, tannins (very low), terpenoids (very low), flavonoids and alkaloids.[291] Etherial, aqueous and ethanolic extracts of seeds and stems (separately) of the plant were prepared, and were for main compound groups (phenolics, flavonoids, alkaloids …etc.). Then they were analyzed by TLC and some known compounds were identified.[292] Essential oil of flowers was extracted by hydrodistillation and analyzed by three sucessive methods, identifying volatiles and odor activity values: β-damascenone was highest.[293] Total phenolic content in aqueous and ethanolic extracts (plant parts not indicated), as well as quantitative analysis of the extracts by HPLC. All known compounds.[294] Qualititaive analysis of fruits by extracting with several solvents and detection of the content of major compound families.[295] Crude fiber content of stems and leaves was determined, along with influences of locality an seasonality. The isolated crude fiber was fed to rat causing an icrease of faecal weight, indicating laxative activity.[296] Whole plant (before flowering) was extracted with acetone/*n*-hexane (1:1) and analyzed by GCMS for poly aromatic hydrocarbons (PAH's) and poly chlorinated benzenes (PCB's), in plants that grew in contaminated soils. PAH's were below detection limit but PCB were accumulated in the plant.[297] Flowers were extracted with methanol and total phenolic content, saponins and alkaloids were determined. The antioxidant of the extract was tested by several methods.[298] Ethanolic extract of flowers was analyzed for total phenolic content, total flovonoids, anthocyanins, carotenoids and fatty acids. Antioxidant activity of the extract was tested (4 methods, high) and hypoglycemic activity (α-amylase and α-glucosidase inhibition, very high)[299]
Polysaccharides and their properties	Leaves were extracted with 80% EtOH/water and extract fractionated to yield polysaccharides.No analysis for monosaccahrides is reported but detailed theoretical is presented for extraction and antioxidant activity of the polysaccharides.[300] Aerial parts was sequentially extracted with *n*-hexane, ethanol and water to yield 9.6% polysaccaride, which consists galactose, glucose, uronic acids, arabinose, and rhamnose (4:5:14:6:1, MW= $1.3x10^6$ KD). The polysaccharide has anti-ulcerative activity in rats.[301] Leaves enzyme (cellulase) assisted extraction of some polysaccharides. Theoretical is presented and antimicrobial, antitumor and antioxidant activities are reported[302]

Nanoparticles, synthesis, uses	Leaves aqueous extract and $CuCl_2$ were used to prepare CuONP's and their antibacterial activity was tested.[303] Leaves aqueous extract and $AgNO_3$ were used to prepare AgNP's and their mosquito larvicide activity was tested. These AgNP's have almost no effect on other aquatic organisms[304]
Antioxidant applications	Aqueous decoction of leaves and flowers was prepared and tested for antioxidant activity (DPPH, NBT) and gainst ammonium metavanadate (NH_4VO_3) kidney toxicity in rats.[305] 80% Methanol/water extract was prepared (plant part not indicated) and its antioxidant activity (DPPH, fenton reagent) was determined. Also, protection of fatty acids was measured *in* vitro[306]
Sedation, anti-anxiety, anesthetic, radiation side effects relief, anti-seizure	Methanol:chloroform (7:3) extract was prepared from stems and leaves. It was tested in rats for sedation, pre-anesthetic and anti-anxiety effect compared with with diazepam (valium, stronger)[307] Patients with radiation therapy of prostate cancer and experiencing severe dysuria, were treated with plant powder (parts not indicated), showing notable improvement of pain relief[308] Plant powder (parts not indicated) were extracted with 85% ethanol/water. The extract was found anti-seizure active in PTZ-induced seizure models on mice[309] Equal portion of the powder of the *M. sylvestris* and *Alcea digitata* (plants parts not reported) were used to treat patients with xerostomia (dry mouth) after radiotherapy of head or neck cancer.[310] Hydroalcoholic extract (no ratio, no plant parts) was used to treat mice with convulsion induced anxiety.[311] Whole plant aqueous extract was found protective against UV-B radiation in mice.[312]
Enzyme related activities	Methanol:water (4:1) extract was prepared (plant parts not indicated) and was tested for angiotensin converting enzyme (ACE) inhibition: moderate.[313] Catalase was partially purified and immobilized onto chitosan and its catalytic properties were studied[314]
Interaction with drugs and toxic compounds	Aerial parts aqueous extract was administered to Leghorn chickens along with Bromhexine HCl. The extract reduced the adverse side effects of the drug.[315] Whole plant was extracted with 95% aqueous methanol and the extract was tested against paracetamol-induced hepatotoxicity and found significantly active[316] Most (87%) patients at basic health units in Brazil reported using *M. sylvestris* products as an alternative and complementary therapy[317] Leaves aqueous extract ameliorated motor asymmetry in rats induced by 6-hydroxydopamine but had no protective effect neurons[318] 50% Methanolic (water) extract was prepared, analyzed for major compound families, minerals and tested for antioxidant activity (2 methods). Also, it was tested for reduction of lithium carbonate damages (oxidation, body weight gain, kidney) in rats: very positive[319] 50% Methanolic (water) extract was prepared, analyzed polysaccharides and tested for Fe(II) chelating and antioxidant activity. Also, it was tested for reduction of lithium carbonate damages (heart, testicles) in rats: very positive[320] Ethanol-water (4:1) extract was tested against sodium fluoride nephrotoxicity and found active[321]
Constipation relief	Flowers aqueous extract was administered to people with constipation and stool problems, Constipations decreased and stool form changed from hard to normal (self reported).[322] Constipation in rats was induced by loperamide and treared with leaves aqueous exreact and yohimbine as control laxative (see Figure 8)[323]
Mouth and throat diseases	A herbal mouthwash consisting of *Althaea officinalis*, *Salix alba* and *M. sylvestris* leaves (ratio, 5:1.25:1, respectively). Leaves extracted with ethanol and the dry extract was suspended with water (0.31%). This suspension was used to treat people with chronic periodontitis and using chlorhexidine as control, and positive results were obtained.[324] Flowers 95% ethanol/water extract was tested for sore throat treatment: inactive[325]
Corrosion prevention	Leaves methanolic with metal corrosion prevention activity was analyzed to determine major compounds responsible for this activity: furfural; levoglucosenone and Levoglucosan, two seven membered multi oxygenated heterocycles; 1,4:3,6-Dianhydroalpha-d-glucopyranose, six membered multi oxygenated heterocycle[326]
Functional foods for pets	Special food that contains human food ingredients suc as *M. sylvestris* was fed to pets to treat atopic dermatitis: positive results[327]
Multiple activity	Leaves ethanolic extract was prepared and fractionated with *n*-hexane, chloroform and ethyl acetate. The extract and fractions were tested for anti-inflammatory, anti-osteoclastogenic and antioxidant activities *in vitro* and *in vivo*[328]

Table 9. Modern research findings of *Malva verticillata*

Property	**Methods and major findings (reference)**
Isolation, analysis, characterization and activities of seed polysaccharides	A novel polysaccharide was isolated and characterized from seeds. It is composed of L-arabinose, D-galactose and D-glucose (3:6:7)[329] Another two novel polysaccharides were isolated and characterized: L-arabinose, D-galactose and D-mannose (14:28:1, MW= 57000); D-glucose, D-galactose and D-mannose (10:1:1, MW= 10400).[330] Peptidolycan was isolated from seeds and analyzed to reslt 43 % polysaccharide and 57 % protein, with total molecular weight of 22000 kD. The detailed amino acid composition is reported (page 2791) and the polysaccharide is composed of L-arabinose, D-xylose, D-galactose, L-rhamnose and D-galacturonic acid (6:5:3:8:24).[331] Branched

	acidic polysaccharide was isolated from seeds. It cosists mainly of arabinose, xylose, galactose and galacturonic acid, and has phagocytic activity.[332] Seven polysaccharides and peptidogylcans were isolated from seeds, and some of them showed anti-complementary and hypoglycemic activities.[333] Novel acidic polysaccharide was isolated from seeds, and it showed phagocytic and anti-complementary activities (L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid (30:15:20:3:2:10, MW= 26000).[334] A neutral, branched polysaccharide was isolated and analyzed by chemical and enzymatic tools (glucose, galactose and arabinose), and it showed phagocytic activity[335] Branched acidic polysaccharide was isolated and analyzed showing anti-complementary activity.[336] Branched acidic polysaccharide was isolated from seeds, and it has phagocytic activity.[337]
Heavy metals accumulation	Compared with three other plants, *M. verticillata* was found to be moderate toxic metals accumulator, even though Cd accumulation was highest in some samples.[338] Out of four plants, *M. verticillata* was found to be good metal accumulator in average, and highets for both Cd and Pb.[339]
Antimicrobial, antidiabetic, antibacterial	Leaves methanolic and n-hexane extract were prepared. Methanolic extract which contained mainly steroids and flavonoids, showed moderate activity against *E. Bacillus*, *S. coli* and *S. aureus*, while hexane extract showed moderate activity against the first two bacteria.340 Seeds were extracted with ethanol followed by fractionation with n-hexane, chloroform, ethylacetate, n-butanol and water. The original extract had significant antidiabetic activity. Among the fractions, the hexanic had the highest activity.[341] Leaves methanolic extract was tested against alloxan-induced diabetes in rats:high. 70 % Methanol (water) leaves extract was tested against eleven bacteria species: inactive.[342] Leaves methanolic extract was tested for glucose-lowering activity in rats:high.[343]
Antioxidant, anti-inflammatory, anti-dermatitis	80 % Methanolic (water) extract (plant part not indicated) was tested for antioxidant activity by two methods (moderate) and total phenolic content (low).[344] Seeds were extracted with seeds or other parts of seven additional plats. The extract found to be anti-inflammatory, heme oxygenase-1 inhibitor and immunomodulatory.[345] Hydrodistillation of leaves yielded an oil that found active against LPS induces dermatitis.[346] Leaves aqueous extract found active against ulcerative inflammation induced by reserpine in mice.[347] Seed ethanolic extract was fractionated with methylene chloride, ethyl acetate and water. The extract and the fractions were tested in Wnt/β-catenin reporter activity assay. It modulated the β-catenin pathway of dermal papilla cells.[348]
Osteoclastogenesis and bone resorption supression	Seed aqueous extract inhibited osteoclastogenesis stimulated by receptor activator of nuclear factor-kB. The extract was analyzed by GCMS and 14 compounds were identified. Authors conclude that medicinal activity should be related to synergims of compounds.[349] (see note after this reference).
New compounds	Seeds were extracted with ethanol followed by fractionation with methylene chloride extract, ethyl acetate and water. Each fraction was analyzed by liquid chromatography and a new compound was isolated: verticilloside, 3-O-[β-D-(6'-linoleoyl)glucopyranosyl]-β-sitosterol (see Figure 9).[351] Leaves were extracted successively with petroleum ether and ethyl acetate. Two new compounds were isolated (Figure 9) and tested for antibacterial activity.[352]

Table 10. Reported heavy/toxic metals accumulation capacity of *Malva* species.

Malva species	Metal(s)	Method	Capacity (reference)
aegyptiaca	Cd(II)	plant from soil	high135
nicaeensis	Zn(II), As(III)	plant from soil	very high[146]
sinesis	Cd(II)	plant from soil	very high[152]
parviflora	general	plant from soil	low[197,198]
parviflora	Zn(II)	plant from soil	medium[214]
parviflora	Cd(II), Cu(II)	plant from soil	high[215]
sylvestris	general	plant from soil	very high[261]
sylvestris	general	plant from soil	medium for Pb(II)[262]
sylvestris	Pb(II), Ni(II)	plant from soil	medium[263]
sylvestris	Pb(II)	dry sieves from aqueous solution	very high[265]
sylvestris	Hg(II)	dry sieves from aqueous solution	very high[266]
sylvestris	Pb(II)	dry sieves with charcoal from aqueous solution	very high[267]
verticillata	general	plant from soil	medium (high for Cd)[338]
verticillata	general	plant from sewage	High for Cd and Pb[339]

If the inconsistency of references 261 and 263 can be ignored, *Malva* species as living plants or as fabricated sieves, can be used for removal of heavy metals from soil or water. It is also clear that *Malva* species are successful cadmium and lead accumulators.

Many of the studies that we cited here report antimicrobial, antibacterial and/or antifungal activities. Among those, it is interesting to notice the contradiction between the data reported in references 270 and 271: while the first reports the antifungal activity of *M. sylvestris* tincture, the second found no antimicrobial activity of the plant tincture, which is a component of commercial mouthwash. Despite this, the antibacterial, antimicrobial and antifungal activities of *Malva* species are proven beyond any doubt (see tables 6-9).

One of the interesting reports of the uses of *M. sylvestris* in herbal-traditional medicine, was published by S. G. Oliviera *et al*. in 2015.[317] They indicated that about 87 % of their study sample of people, reported the use of this plant and its products along with allopathic medicines or without them. Authors warn (page 5) that people are unaware of the plant possible toxicity, citing M. R. Ritter *et al*.[370] But reading carefully the publication of M. R. Ritter shows that there is no mentioning of *M. sylvestris* toxicity. Moreover, it was shown by Consolini & Ragone, that this plant is completely safe for all potential uses.[371]

In recent decades, there is growing interest in reducing damages of synthetic drugs by using natural plant extracts or other plant products. Among these synthetic drugs and chemicals, both organic, but mainly inorganic chemicals are widely used, and have some severe adverse effects.[372,373] A. A. Ben Saad and his colleagues, presented very promising results of using *M. sylvestris* aqueous extract to reduce the side effect of lithium carbonate.[319,320] Similar reports were published by other groups. For example, petroleum ether extract of *Solanum trilobatum* has a protective effect against lithium carbonate,[374] but unlike *M. sylvestris* that is soft to touch and completely safe,[375] *S. trilobatum* is a thorny plant and mildly toxic. The same Tunisian group of A. Ben Saad *et al*. is searching for other plants that can ameliorate damages of lithium carbonate, and recently they have published the positive results of their study of juice of *Opuntia ficus-indica* (cactus) thorny cladodes.[376]

Use of *Malva* species for constipation relief is known in most traditional medicines of Asia (*M. neglecta*[36,41,52,59], *M. parviflora*[67] and *M. sylvestris*[108,110,115]). This activity is recently investigated by several groups. One of the interesting reports was published by a M-A. Jabri and his colleagues.[323] They induced constipation in rats by loperamide (Figure 8), a synthetic alkaloid, usually marketed as the commercial Imodium for diarrhea relief.

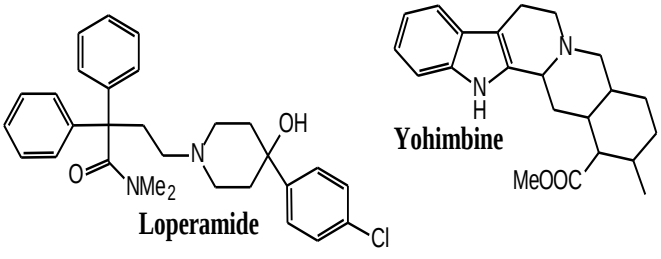


Figure 8. Structures of loperamide andf yohimbine[323]

Constipation was successfully treated with aqueous extract of *M. sylvestris* leaves. As control laxative, they used yohimbine (Figure 8), naturally occurring major alkaloid found in the bark of *Pausinystalia johimbe*.[377]

The anti-constipation activity of *M. sylvestris* leaves extract explained by the authors as a result of the antioxidant capacity, and they present possible mechanism of action (page 6). M. Elsagh *et al*. reported the successful use of flowers aqueous extract for the same goal.[322] Since both leaves and flowers of this plant are flavonoid-rich, these results of these two reports are consistent.

The presence of polysaccharides in *Malva* species drew the much attention from research groups. The isolated and characterized (fully or partially) polysaccharides were also tested for various activities viz., *M. aegyptiaca*,[133] *M. mohileviensis* (anti-inflammatory, antioxidant),[143] *M. pussila* (anti-inflammatory),[149] *M. sylvestris* (anti-complementary,[220] antioxidant,[222] anti-ulcerative, antimicrobial, antitumor and antioxidant[300-302]). All polysaccharides in these species are acidic, containing glycoronic acids, and relatively, water soluble. Polysaccharides isolated from seeds of *M. verticillata* are neutral,[329,330] and acidic with high acid content.[331] Later on, this Japanese group reported that some of these acidic polysaccharides (or peptidoglycans) have medicinal activities such as phagocytic,[332,337] anti-complementary and hypoglycemic,[333] phagocytic and anti-complementary,[334] and anti-complementary.[336] Phagocytic activity was also found in the neutral polysaccharide.[335] This extensive study that was done with *M. verticillata* should be done with other common *Malva* species.

Treating hair loss with *Malva* species products is known from traditional medicine of Iraq (*M. parviflora*),[73] and Iran (*M. sylvestris*).[115] Modern research confirmed these uses and E. Y. Lee *et al*. report that *M. verticillata* ethanolic extract and its fractions are modulating the Wnt/β-catenin pathway of dermal papilla cells, and thus, it's a good candidate for treating hair loss.[348] Their analysis revealed that the active compound is myristoleic acid (C14: 1 *cis* 9).

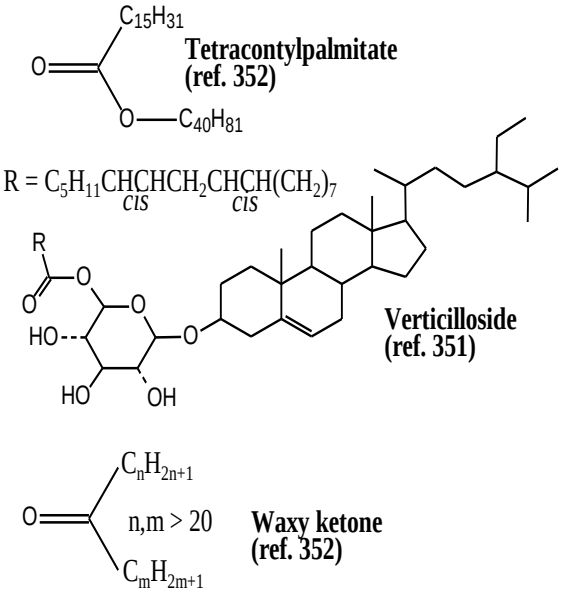


Figure 9. Structures of verticilloside[351] and two waxy compounds[352]

Another medicinal property that is known to traditional medicine and was confirmed lately by modern research is the ability of *Malva* extracts to repair bones. *M. neglecta* is used in traditional medicine of India (leaves),[26] and Turkey (whole plant),[41] to repair fractured bones. K-S. Shim *et al*. from Korea, report that water extract of *M. verticillata* suppresses osteoclastogenesis and bone resorption.[349]

M. verticillata is still providing modern research with new and very interesting compounds. One of these compounds was isolated and characterized by and her colleagues from ethanolic extract of the plant: verticilloside (Figure 9).[351]

Another two new compounds were isolated from leaves extract: tetracontanyl palmitate ($C_{56}H_{112}O_2$) and a waxy ketone (Figure 9), but the exact structure of the ketone was not completely elucidated.[352]

Conclusions

While studying the vast mass of publications about the genus *Malva*, we reached some important understandings that must be conveyed to interested readers. Some of these publications are inaccurate, to say the least (reference 71 and others). Moreover, some publications do not report any new findings of plants of *Malva* genus. M. Ishtiaq and his colleagues report an ethnomedicinal survey of the plants of Samahni valley in Pakistan.[378] In fact, there is no new information at all about *M. parviflora*, which is listed there, and for this reason, it was not included in table 2. Same consideration was taken for the report of Jeambey et al from Lebanon.[379] They cite previous reports and plants consumption as food (*M. sylvestris*), but again, no real information was provided concerning health or medicinal use of this plant.

During the writing itself, we discovered a strange fact: despite being one of the most useful species of *Malva*, *M. nicaeensis* was very poorly studied for medicinal-biological activities. It is widespread in the Middle East, especially in the Western part of it, and it is used as food in various ways (see table 4). It is also worth noticing that compared with other major Malva species, its traditional medicinal uses were very partially reported. Such reports were lately published.

M. Mosaddegh and his colleagues from Iran report that its roots and flowers decoction is traditionally used to treat stomach pains.380 Nasab & Khosravi (Iran) present the ethnomedicinal use of M. nicaeensis, along with M. parviflora and M. sylvestris.381 Its fruits decoction is used to treat cold and sore throat. S. Baydoun and her colleagues from Lebanon report that leaves decoction to have several uses to treat: catarrhs, renal infections, kidney stones, respiratory infections, constipation and skin diseases.[382] Strangely enough, only three reports were published about medicinal activities of this plant,[146-148] and its chemical composition, partial or complete, was never published. Moreover, for other major species, some same activities were reported by many groups or the same group more than once: antioxidant, antibacterial, anti-inflammatory. These activities were never published for *M. nicaeensis*.

Our group is currently working on these studies.

Therefore, we conclude and recommend:

(1) There is a need for extensive, systematic study of *M. nicaeensis*.
(2) Contradicting reports of medicinal activities of *Malva* species must be resolved by follow up studies.
(3) Proven and very useful medicinal activities of *Malva* species (antidiabetic, antiobesity) must draw more attention of researchers to convert this potential to practical drug treatments.
(4) It is very important to re-evaluate contradicting and inaccurate reports by further studies.
(5) It is important and useful to isolate the natural product/s responsible for medicinal activities of the plants, and study the mechanism of action. This can open a window of improving the activity.

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Received: 11.06.2017.
Accepted: 12.07.2017.

SPECTROSCOPIC STUDIES OF NEWLY SYNTHESIZED STEROIDAL DIHYDROPYRROLES

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Keywords: Steroidal oxime, hydroxylamine, dihydropyrrole, vinyl chloride.

A convenient procedure for the synthesis of 3β-acetoxy-3′-chloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole (**4**), 3β,3′-dichloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole, (**5**) and 3′-chloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole (**6**) has been made from steroidal oximes (**1-3**) under refluxing conditions. The structural assignment of the products was confirmed on the basis of IR, ^{1}H NMR, 13C NMR, MS and analytical data.

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Introduction

Pyrrole derivatives are ubiquitous among naturally occurring organic compounds.[1] They are vital building blocks for the construction of bio-active molecules such as porphyrins, alkaloids and co-enzymes.[2] The biological importance of pyrrole and its derivatives cannot be overemphasized because they have shown to possess extensive biological activities and pharmacological properties such as antimicrobial, analgesics, ionotropic, antitumor, anti-inflammatory, and antiallergic.[3-10] These have also been employed as P38kinase,[11] prolyl -4-hydroxylase,[12] poly(ADP-ribose) polymerase inhibitors,[13] estrogen receptor β-selective ligands,[14] AT1-selective angiotensin II receptor antagonists,[15] and minor groove recognition elements.[16] Several macromolecular antibiotics having pyrrole structure were isolated from biological sources, and their activities were defined.[18,19]

In the view of their importance, the synthesis of pyrroles itself remains an attractive area. And there is a continuing interest in developing versatile synthetic routes.[20] In the light of the previous introductory comments, it is not surprising that a vast amount of work has and is being devoted to the development of practical methods for the synthesis of pyrrole building blocks containing appropriate substitution patterns.[21] However, a search of the literature for effective methods for the synthesis of pyrrole libraries to be employed in high-throughput screening remains a challenge for medicinal chemists.

Although construction of pyrrole ring has been done by several methods, for example, the Knorr,[22] Paal-Knorr,[23, 24] and Hantzsch syntheses.[25] [3 + 2]-cycloadditions,[26-28] multi-component reactions,[29-31] and ring contractions[32] or cyclizations,[33-36] a novel and efficient synthetic method for the synthesis of pyrroles is currently being pursued. Unfortunately, the usual reaction conditions are extremely harsh, requiring strong bases/high temperatures and unselective products. These factors limit the overall synthetic utility of above-mentioned methods.

Keeping all the above applications of pyrroles in consideration and in connection with our previous work[37] aimed at developing a convenient synthetic strategy, we sought to develop a milder reaction condition to broaden the scope of this important transformation. We wish to report herein, an efficient one-pot procedure for the conversion of 5α-cholestane-6-one oximes (**1-3**) into 3′-chloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrroles (**4-6**) using p-TsOH and vinyl chloride in acetonitrile under reflux conditions.

Experimental

General Methods

The IR spectra were recorded in KBr on Pye Unicam SP3-100 spectrophotometer and its values are given in cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with tetramethylsilane (TMS) as internal standard and its values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapours to check the homogeneity as well as the progress of the reaction. Sodium sulfate (anhydrous) was used as a drying agent. All chemicals used in this work were purchased from Merck India.

Synthesis of 3′-chloro, 5α-cholest-6-eno [7, 6 - *d*] 2′, 3′-dihydro-1H-pyrroles (4-6)

To a mixture of 5α-cholestane-6-one oxime **1-3** (1 mol) and p-TsOH (0.5 mg) in CH_3CN (20 mL), was added vinyl chloride (1 mol) in the same solvent and reaction mixture was refluxed for 7 h. The progress and purity of the reaction were checked by TLC. After the completion of the reaction, excess solvent was removed to three-fourths of the original volume under reduced pressure. The reaction mixture was taken in diethyl ether (15 mL) and washed with water (40

mL) thrice successively and dried over anhydrous sodium sulfate. Removal of solvent and crystallization from methanol provided the desired product **4-6**.

3β-Acetoxy-3′-chloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole (4)

Yield (73 %); Anal. Calc. for $C_{31}H_{50}NO_2Cl$: C, 73.85, H, 10.01, N, 2.78. found: C, 73.73, H, 9.96, N, 2.70; IR (υ cm^{-1}): 3270 (N-H), 1735 (O*CO*CH_3), 1620 (C=C), 1080 (C-O), 1105 (C-N), 742 (C-Cl); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.5 (s, 1H, N*H*, exchangeable with D_2O), 4.6 (m, 1H, C_3α-*H*, $W_{½}$ = 15Hz), 3.9 (m, 1H, C_3′*H*Cl, $W_{½}$ =17 Hz), 3.3 (d, 2H, C′H_2), 2.01(s, 3H, OCOCH_3), 1.12 (s, 3H, C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.90 & 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171, 132, 128, 72.8, 52, 50, 42 40, 37, 29, 27, 26; ESI-MS: *m/z* 503/505 [$M^{+\cdot}$].

3β,3′-Dichloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole (5)

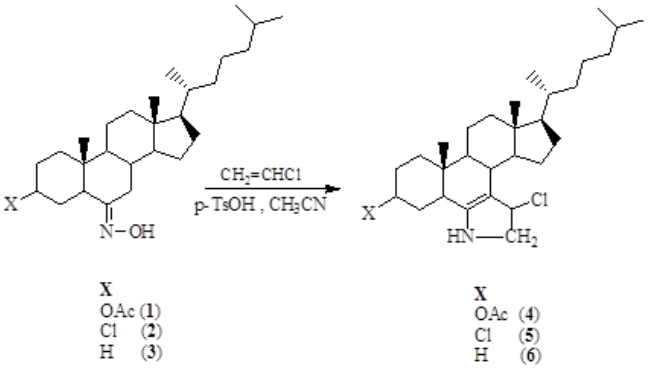
Yield (70 %); Anal. Calc. for $C_{29}H_{47}NCl_2$: C, 72.47, H, 9.86, N, 2.91. found: C, 72.39, H, 9.79, N, 2.88; IR (υ cm^{-1}): 3260 (N-H), 1622 (C=C), 1110 (C-N), 750, 744 (2×C-Cl); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.7 (s, 1H, N*H*, exchangeable with D_2O), 4.3 (m, 1H, C_3α-*H*, *W*½ = 15 Hz), 3.7 (m, 1H, C_3′*H*Cl, $W_{½}$ =17 Hz), 3.5 (d, 2H, C′H_2), 1.12 (s, 3H, C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.90 & 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 130, 127, 54, 51, 42 40, 37, 29, 27, 26; ESI MS: *m/z* 479/481 [$M^{+\cdot}$].

3′-Chloro-5α-cholest-6-eno[7,6-*d*]-2′,3′-dihydro-1H-pyrrole (6)

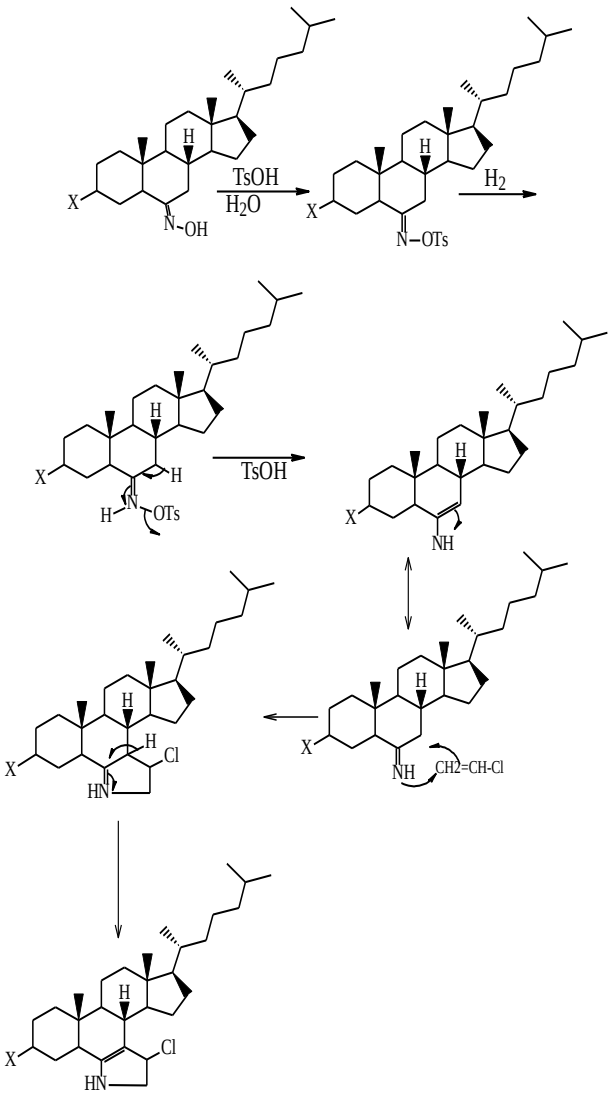
Yield (75 %); Anal. Calc. for $C_{29}H_{48}NCl$: C, 78.07, H, 10.84, N, 3.14. found: C, 77.96, H, 10.81, N, 3.11; IR (υ cm^{-1}): 3275 (N-H), 1628 (C=C), 745 (C-Cl), 1120 (C-N); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.5 (s, 1H, N*H*, exchangeable with D_2O), 3.9 (m, 1H, C_3′*H*Cl, $W_{½}$ = 17 Hz), 3.4 (d, 2H, C′H_2), 1.12 (s, 3H, C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.90 & 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 132, 128, 52, 50, 42 40, 37, 29, 27, 26; ESI MS: *m/z* 445/447 [$M^{+\cdot}$].

Results and discussion

The substrates employed for initial studies are 3β-acetoxy-5α-cholestan-6-one oxime **1b** (*m/z* 459)**,** 3β-chloro-5α-cholestan-6-one oxime **2** (*m/z* 435) and 5α-cholestan-6-one oxime **3** (*m/z* 401) which were synthesized by the treatment of steroidal ketones[38] with $NH_2OH.HCl$ and sodium acetate trihydrate in ethanol under reflux conditions.[39] The oximes **1-3,** when allowed to react with vinyl chloride in an acidic medium under reflux conditions, afforded steroidal dihydropyrroles **4-6** (Scheme 1). The products have been characterized on the basis of their elemental and spectral studies.



Scheme 1. Schematic representation of the formation of steroidal pyrrole derivatives



Scheme 2. Mechanistic outline for cyclization of steroidal oxime into steroidal dihydropyrroles

All the compounds **4-6** exhibited IR absorption bands at 3260-3275 cm^{-1} (N-H), 1105-1120 cm^{-1} (C-N) and 1620-1628 (C=C) which suggested the formation of dihydropyrrole ring in the products. Further, stretching vibration at 742-750 (C-Cl) is attributed to the chlorine attached to the dihydropyrrole ring. The formation of steroidal pyrroles was further confirmed on the basis of ^{1}H NMR spectra. Assignments of the signals are based on the chemical shift and intensity pattern. The ^{1}H NMR spectra of the compounds exhibited a singlet (exchangeable with D_2O) for one proton (N*H*) at δ 7.5-7.7. It also predicts multiplet for one proton at δ 3.3-3.5 suggested the presence of C_3′H-Cl. ^{13}C NMR signals are in good agreement with the proposed structure of synthesized compounds.

All the compounds show δ 51-56 (*C*-Cl) which are attributed to the presence of chlorine. The signal in the range of 120-147 (C=C) and 46.5-46.8 (*C*-N) confirm the presence of pyrrole ring. The plausible mechanism[40] of this conversion is shown in Scheme 2. This conversion involves the initial reaction of steroidal oxime with *p*-toluenesulphonic acid to form the o-tosyl derivative. Protonation on nitrogen and subsequent removal of *p*-TsOH gives enamine intermediate. This upon reaction with vinyl chloride affords desired product **4-6**.

Conclusion

In conclusion, we have demonstrated an efficient and facile synthesis of steroidal dihydropyrrole derivatives in a one-pot operation from steroidal ketoximes. Moreover, this methodology offers significant advantages with regard to simplicity of operation, the yield of products, easy workup and mild reaction conditions. It provides a better alternative for the synthesis of dihydropyrroles. In short, the present procedure would shed new light on the convenient approach for the preparation of dihydropyrroles.

Acknowledgements

Authors thank the Chairman, Department of Chemistry, A.M.U., Aligarh, for providing necessary research facilities. Facilities provided by SAP (DRS-I) for their generous research support are also gratefully acknowledged. Authors SM and MJ also thank Head department of Chemistry OPJS University for research support.

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Received: 14.06.2017.
Accepted: 15.07.2017.

QUALITY STATUS OF DOXYCYCLINE IN TABLETS

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Keywords: Doxycycline, generic, quality control, interchangeability, tablets.

Doxycycline is a second-generation semi-synthetic antimicrobial of tetracyclines family. It is a broad spectrum antimicrobial agent used in several countries for the treatment of diseases such as chronic prostatitis, sinusitis, syphilis, chlamydia, pelvic inflammatory disease as well as additives in animal feed to improve its growth. Doxycycline is distributed free of charge through Unified Health System in Brazil, which, according to its acquisition, for subsequent distribution, does not always guarantee the same brand. Five different brands of doxycycline tablets (the reference pharmaceutical of doxycycline, two generic and two similar products) were used for evaluation of the interchangeability of the tablets by determination of average weight, hardness, friability, disintegration, the active principle content, content uniformity and in vitro dissolution. All tablets submitted to the tests of average weight, friability, hardness, and disintegration were according to specification. Tests of content uniformity show drugs out of specification, with contents higher than 105%. In the trial of dissolution, there were no statistical differences in the profiles. Due to the results obtained, all doxycycline tablets analyzed should not be approved by the Quality Control authorities. Sectors of production, analytical development, and quality control should meet to resolve this issue. Thus, the importance of a pharmaceutical equivalence study is important to guarantee a safe interchangeability and consequently the same therapeutic effect.

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INTRODUCTION

Doxycycline (DOX) is a second generation semi-synthetic antibiotic of tetracyclines family.[1] DOX is an antibiotic used for treatments of diseases caused by anaerobic and aerobic bacteria[2] as well as Gram-negative and Gram-positive bacteria.[3]

DOX is, preferentially, chosen when compared to the other tetracycline in specific infections due to the high absorption and its long half-life which allows a lower dosage frequency[4] and to have a better clinical efficacy in low concentrations, such as between 2 or 4 times the level of minimal of inhibition concentration (MIC) for susceptible microorganisms. Therefore, the inhibition of microorganisms by the drug occurs in a time-dependent manner.[5]

In Brazil, the public health system is called the Unified Health System (UHS) and distributes free and low-cost medicines to the population. The drugs that are distributed conform to the local epidemiological profile and aim for therapeutic gains. However, the UHS also aims economic controls, and therefore, procurement of distributed drugs is done by an acquisition process. This process is intended to ensure compliance with the constitutional principle of isonomy and to select the most advantageous proposal for Public Administration. The best options, presented by the suppliers, will be acquired by UHS bringing a variation of brands of the medicines available. Thus, it is evident the need for an antimicrobial quality control in order to guarantee a quality standard, as well as its effectiveness and safety since the population has easy access to the antibiotic and there are variations of the brands.

DOX are found in tablet forms, oral liquid formulations, and capsules in Brazil.

In the market, there are currently generic and similar forms of doxycycline tablets. The company that developed and markets the reference product is Pfizer under the trade name Vibramycin™.

According to the National Agency of Sanitary Surveillance (ANVISA), reference products are those innovative products registered at the federal agency responsible for sanitary surveillance and marketed in the country whose effectiveness, safety and quality have been scientifically proven by the competent federal agency at the time of registration, as is defined in item XXII, article 3, Law. 6,360, of 1976, as amended by Law. 9,787 of February 10, 1999.[6]

Generic medicines are those containing the same active principle, at the same dose and in the same pharmaceutical form, administered in the same way and with the same dosage and therapeutic indication of the reference medicine, presenting efficacy and safety equivalent and which may be interchangeable.[6]

Finally, similar medicines are those containing the same or the similar active principles, have the identical concentration, pharmaceutical form, route of administration, dosage and therapeutic indication, and which is equivalent to the medicine registered in the federal agency responsible for health surveillance, differ only in characteristics related to the size and shape of the product, shelf-life, packaging, labeling, excipients and vehicle, and should always be identified by trade name or brand.[6]

It is important to emphasize that generic and similar drugs must be interchangeable with reference medicines, it means, they have to present pharmaceutical equivalence - same drug, same dose or concentration and must comply with the

same in vitro specifications, bioequivalence and dissolution profiles compared with the reference ones in order to obtain therapeutic equivalence between them and be possible to substitution by generic or similar products.[6]

If there is no interchangeability between the medicines, the clinical efficacy and safety will not be the same, the treatment of the patient will be compromised and generate a public health problem.

Therefore, the quality control of drugs and medicines is very important, it ensures that the drug has the capacity to exert the therapeutic effect that is expected. Thus, the study on the pharmaceutical equivalence of DOX is extremely relevant, since it does form the part of the list of medicines of the UHS with easy access to the population through medical prescription and due to its acquisition of by UHS. In addition, the pharmaceutical form which DOX is distributed to the population is coated tablet, therefore, solid medicament, and since these may present greater problems with regard to bioavailability, it becomes relevant to evaluate the impact of these factors on the dissolution of the drug in the pharmaceutical form, performing an in vitro test that allows visualizing how its dissolution occurs as a function of time.[7]

This study aimed to evaluate the quality and the equivalence of doxycycline tablets currently sold, by determining the average weight, hardness, friability, disintegration, the active principle content, content uniformity and in vitro dissolution of the reference medicine of doxycycline, two generic and two similar products.

EXPERIMENTS

The adjuvants contained in the dosage form were doxycycline coated tablets containing 80 mg (labeled content) of Similar A and 100 mg (labeled content) of Similar B, Generic C 100 mg (labeled content), Generic D 100 mg (labelled content), and Reference 100 mg (labelled content). The raw material was doxycycline, content 97.10 %, lot 0900002795, kindly provided by União Química Pharmaceutical Industry (São Paulo, Brazil). All chemicals used were of pharmaceutical grade.

Determination of average weight

Twenty randomized tablets of doxycycline of each sample were individually weighed in Mark's semi-analytical balance (Bel Engineering™).

Hardness test

Ten randomized tablets of each brand were tested to determine their radial crushing strength by model 298-AT durometer (Nova Ética™).[8,9]

Friability test

Twenty doxycycline tablets of each brand were used in order to obtain the percentage of friable particles by model 300-1 friabilometer (Nova Ética™).[8,9]

Disintegration test

Six tablets of each sample were used for the disintegration test within the time limit specified by the Brazilian Pharmacopoeia[8] and USP.[9]

Uniformity of content

One tablet at a time was crushed, weighed and analyzed. The equivalent of 5 mg of doxycycline standard was working sample of the Similar sample A, Similar doxycycline B, Generic doxycycline C, Generic doxycycline D and Reference doxycycline. The crushed tablet mass was dissolved and transferred to a 50 mL amber-coloured volumetric flask. From this, aliquots of 750 µL were transferred by the use of automatic pipettes, to a 5 mL amber-coloured volumetric flask to obtain a concentration of 15µg mL^{-1}. A method of estimation was also developed using the UV-1800 spectrophotometer (Shimadzu™) and 1.0 cm quartz cells.

Content

The assay was performed using a crushed tablet pool, which was weighed and analyzed. The equivalent of 5 mg of doxycycline standard the Similar sample A, Similar doxycycline B, Generic doxycycline C, Generic doxycycline D and Reference doxycycline was weighed. A spectrophotometric method was developed using the UV-1800 spectrophotometer (Shimadzu™).

The mass of the crushed tablets was dissolved and transferred to a 50 mL amber-coloured volumetric flask. From this, aliquots of 750 µL were transferred by using automatic pipettes, to a 5 mL amber-coloured volumetric flask to obtain a concentration of 15µg mL^{-1}.

Dissolution test

The dissolution test was performed according to the methodology recommended by USP 37.[9] The test was performed using Technologies 8000 Dissolution sampling Station VK7025 dissolver (Varian™), apparatus II (paddle) under stirring speed 75 rpm and 900 mL of 0.01 M hydrochloric acid at 37 ± 0.5 °C. Aliquots of 10 mL were collected at pre-determined time intervals of 5, 10, 15, 20, 30, 45, 60, 75 and 90 min. After the removal of each aliquot, the medium was not replaced, but this was discounted in the calculations. The cumulative percentage of drug release, determined by reading the absorbance in a spectrophotometer, was plotted against time, in order to obtain the release profile and calculate the in vitro dissolution data (n = 6).

RESULTS

Average weight

According to the Brazilian Pharmacopoeia,[8] film-coated tablets with an average weight of 250 mg or more have a limit of variation up to 5 % and those with sugar coatings (dragees) with an average weight between of 150 mg, and

300 mg have a variation limit up to 7.5 %. Similar and generic drugs fall into the classification of coated tablets and reference into sugary tablets, and none have exceeded the specified limits.

Hardness

According to the Brazilian Pharmacopoeia,[8] the result on the hardness test should be expressed with the average of the values obtained in the determinations and the unit of force (*N*). The results of the analyzed drugs are contained in table 1.

Table 1. Hardness of the tablets

Tablet no.	Force (*N*)				
	A	B	C	D	Ref.
1	68.65	105.91	142.20	114.74	111.80
2	65.70	117.68	129.45	136.31	106.89
3	91.20	90.22	182.40	114.74	104.93
4	71.59	88.26	140.24	148.08	106.89
5	68.65	86.30	129.45	157.89	94.14
6	72.57	79.43	145.14	135.33	109.85
7	67.67	94.14	138.27	166.71	96.11
8	68.65	97.09	133.37	154.95	108.85
9	69.63	90.22	113.76	161.81	129.45
10	58.84	108.85	155.93	166.71	105.91
Average	70.32	95.81	141.02	145.73	107.48
SD	8.24	11.70	18.35	19.71	9.58
RSD (%)	11.72	12.22	13.01	13.52	8.91

Ref. = Reference

Friability

The total mass of the 20 tablets of each sample used in the test, as well as its final mass and possible loss of friable particles are given in Table 2.

Table 2. Masses of DOX tablets before and after the friability test.

Sample	Friability			
	Weight (g)		Difference	
	Initial	Final	(g)	(%)
A	3.6613	3.6613	0	0
B	2.7529	2.7521	0.0008	0.0291
C	5.4318	5.4318	0	0
Generic D	7.3566	7.3500	0.0066	0.0897
Ref.	5.0137	5.0106	0.0031	0.0618

Disintegration

According to the Brazilian Pharmacopoeia,[8] the time limit established as the general criterion for the disintegration of film-coated and sugar-coated tablets is 30 min and 60 min, respectively. The time each drug took for total disintegration is shown in table 3, and at the end of the test, no residue from the tested units remained on the metal screen of the disintegrating apparatus except for insoluble tablet coating fragments.

Uniformity of content

The uniformity test was performed with unit doses. The results of the test are given in table 4.

Content

The dosing was performed with a pool of crushed tablets. The results of the test are given in table 5.

Table 3. Total time for the disintegration of doxycycline tablets.

Sample	A	B	C	D	Ref.
Disintegration time (min)	27	25	28	18	10

Table 4. Determination of content uniformity of DOX tablets by the spectrophotometric method in the UV region.

Sample	A	B	C	D	Ref.
Content (%)	120.12	110.72	121.19	123.56	118.96
RSD (%)	1.83	1.10	1.67	3.06	0.95

RSD = = relative standard deviation

Table 5. Determination of dosing of DOX tablets by the spectrophotometric method in the UV region.

Sample	Tablets DOX content		Content (%)	RSD (%)
	Average ABS DOX standard	Average ABS DOX		
A	0.569	0.689	121.15	1.98
B	0.555	0.629	121.16	1.28
C	0.546	0.644	117.94	1.07
D	0.557	0.650	116.69	2.96
Ref.	0.540	0.641	120.54	1.41

Dissolution

The dissolution test was performed with six tablets of each example, the solvent being 900 mL of 0.01 M HCl, paddle rotation of 75 rpm at 37 ± 5 °C. The absorbance of aliquots of 10 mL drawn at different intervals of time, at 268 nm, was determined. As there was no replacement of solvent, the calculations were discarded, and the release of drug was determined from the line obtained in the analytical curve (Figure 1).

DISCUSSION

Quality control plays a key role in all stages of the production of a drug. The proper analysis of raw materials, intermediate products, and the finished product, associated with the proper control of production processes is essential

for the efficient and safe quality of the product. Adequate verification of the physicochemical characteristics allows a greater quality and therapeutic efficacy.[10]

The quality control made during and after the production of each batch is essential to ensure that the qualities of the product are met.[10] Among the general methods applied during and after production are average weight, friability, hardness, disintegration, and dissolution.

Determining the average weight is intended to ascertain whether the units of the same batch have a uniform weight.

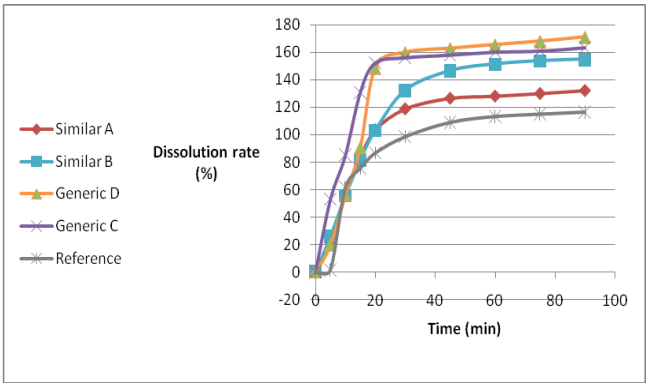

Figure 1. Dissolution profiles of doxycycline tablets.

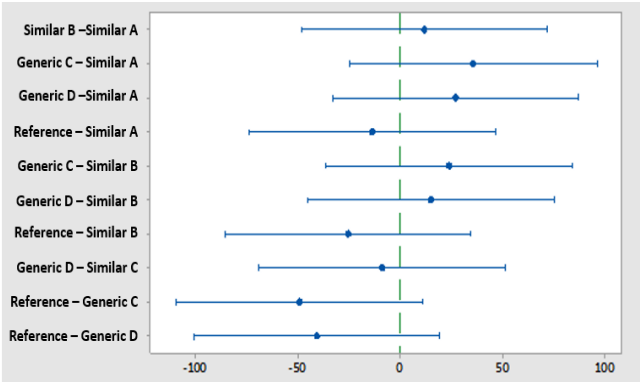

Figure 2. Paired comparison of Tukey for the dissolution profiles of reference, A, B, C and D tablets.

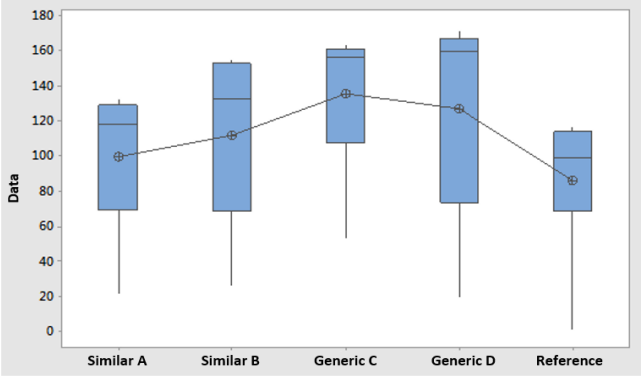

Figure 3. Box plot analysis of the dissolution profiles of different doxycycline tablets.

As the similar doxycycline, A and B, generic C and D are coated or film-coated tablets with an average weight of 250 mg or more, the prescribed tolerance limit is ±5 % of the average weight. However, the reference drug is of the category of sugar-coated tablets and limits of variation for such tablets of 150-300 mg weight is 7.5 %.[8] In this test, no tablet of any brand exceeded the limits specified by the Brazilian Pharmacopoeia.[8]

The hardness test enables to determine the strength of the tablet to the crushing or rupture under radial pressure. The result of the hardness test is only informative and the force exerted must be in expressed in Newton.[8] Therefore, the data should only be compared with the reference medicine. Therefore, the similar drugs have hardness with lower values, and the generics have hardness with higher values when compared to the reference, and this difference of values depends on the adjuvants contained in each formulation and technological procedure.

The friability test allows determining the resistance of the tablets to the abrasion when submitted to the mechanical action at specific apparatus. No tablet must be present at the end of the test chipped, cracked or broken. Tablets with the loss of 1.5% or less of their weight[9] are considered acceptable. In the case of the doxycycline tablets analyzed, in some cases, there was no loss of mass, and in others, there were losses but within the limits prescribed by the Brazilian Pharmacopoeia.[8]

The disintegration test checks whether tablets and capsules disintegrate within the specified time limits, which according to the Brazilian Pharmacopoeia,[8] are 30 min and 60 min for sugar- and film-coated tablets respectively. At the end of the test, all the tablets had disintegrated completely. The disintegration test time for all samples was less than the time specified by the Pharmacopoeia.

In the development of the analytical curve for DOX, concentrations ranging from 0.5 to 60 μg mL^{-1} were explored. Concentrations of 6 to 21 μg mL^{-1} were chosen since they have the linearity of response. After that, a graph of concentration versus absorbance was constructed. The equation was $y = 0.0382x + 0.0013$ with a correlation coefficient of 0.9999, and it was possible to determine the content of doxycycline present in the tablets in the dissolution test.

The spectrophotometric determination in the UV region used in these analyses was previously validated by Kogawa and Salgado.[11]

The content uniformity test, as well as the dosing test, aims to demonstrate the amount of DOX present in the samples analyzed and according to the official specification should be between 95.0 and 105.0 %.[12] All the analyzed samples are in disagreement with the specification, presenting contents higher than 105 %. Hence these tablets should be disapproved by Quality Control.

Dissolution studies are indispensable in the production stages of a drug. With the dissolution characteristics, it is possible to predict the in vivo behavior of the pharmaceutical forms, leading to the reduction of the costs and work required to develop a pharmaceutical form, as well as to the number and size of the required clinical studies.[13] The dissolution test is a physical test in which the percentage of drug dissolved versus time is evaluated.

Analysis of the dissolution profiles of the doxycycline reference tablets, similar A, similar B, generic C and generic D, through the Analysis of Variance, Tukey and Boxplot, showed no statistically significant difference between them.

Thus, doxycycline reference tablets, similar A, similar B, generic C and generic D may be interchangeable.

However, unfortunately, all doxycycline tablets analyzed, reference, similar A, similar B, generic C and generic D should not be approved by the Quality Control sector because of the dosage values and content uniformity found. All of them presented values above those specified.

Sectors of production, analytical development and quality control should meet to resolve this issue. Involvement of all the sectors is vital to achieve the desired goals.

To summarize, the parameters evaluated are important and critical to making the certificate of equivalence between samples.

This study was designed to introduce the concern to the several aspects (planning, producing and controlling).

It is important to note that these products are commercialized or distributed as equivalent, and they were approved by industrial, pharmaceutical quality control.

In order to guarantee the production of high-quality products for both animal and human use, it is essential that all production steps and producers are maintained through the strict observance of standard operations rules.

CONCLUSION

Due to the results obtained, all doxycycline tablets analyzed should not be approved by the Quality Control sector, the interchangeability is not satisfactory. Sectors of production, analytical development and quality control should meet to resolve this issue. Thus, the importance of a pharmaceutical equivalence study is fundamental to guarantee a safe interchangeability and consequently the same therapeutic effect.

ACKNOWLEDGMENTS

The authors acknowledge FAPESP (São Paulo, Brazil) and CNPq (Brasília, Brazil).

Received: 05.06.2017.
Accepted: 19.07.2017.

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RP-HPLC DETERMINATION OF PARACETAMOL-CONTAINING COMPONENTS IN QUATERNARY AND BINARY MIXTURES

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Keywords: use the Keywords style for the list of keywords, separating with a comma each items.

A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) technique for the simultaneous determination of ascorbic acid (ASC), methionine (MET), paracetamol (PAR) and caffeine (CAF) has been developed and validated. The cited components are separated completely using Brownlee Bio C18 column (250 x 4.6 mm, 5 µm) by isocratic elution of water-acetonitrile (85:15) (v/v) mobile phase flowing at 1.0 mL min^{-1} at ambient temperature. The spectrophotometric detection is carried out sequentially at 260 nm for ASC (2 min), 200 nm for MET (1 min), 240 nm for PAR (1.5 min) and 270 nm for CAF (1.5 min). Total chromatographic analysis time per sample was approximately 6 min. The linear range of determination for ASC, MET, PAR and CAF are 40-160 µg mL^{-1}, 40-200 µg mL^{-1}, 20-400 µg mL^{-1} and 40-160 µg mL^{-1}, respectively. Thus, proposed method can be successfully applicable to analysis the pharmaceutical preparation containing the above mentioned drugs without any interference of excipients. Recovery ranges and relative standard deviation are in turn 96.46 to 102.70 %, 2.65 % for ASC, 96.33 to 103.43 %, 2.93 % for MET, 98.31 to 102.73 %, 2.09 % for PAR and 95.82 to 102.13 %, 2.68% for CAF.

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Introduction

Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the following advantages. Speed (analysis can be accomplished in 20 min or less), greater sensitivity (various detectors can be employed), improved resolution (wide variety of stationary phases), reusable columns (expensive columns but can be used for many analysis), ideal for the substances of low volatility, easy sample recovery, precise and reproducible, and easy of automation.

Reversed phase liquid chromatography is generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence travel faster through the column of non-polar stationary phase and take less time to elute. This is because of the lower affinity between the polar compounds and the stationary phase. In chromatographic analysis, the main problem of this method involves the optimization of experimental conditions such as selection of column type, temperature of column, variety and composition of mobile phase, selection of specific wavelength and cheap instrumentation. In spite of the fact that this method undoubtedly provides more sensitive determination than the spectrophotometric methods, Reversed Phase Chromatography (RPC) is effective, reproducible and rugged and often easier for UV detectors. It has now become the method of choice for most of drug and combinations of drugs.[1,2]

The objective of using this method in the present study is to develop and validate a specific, accurate, precise and reproducible quality control method for ASC, MET, PAR and CAF in their quaternary and binary combinations.

Experimental

Chemicals and Reagents

All chemicals are of HPLC-analytical grade and are used without further purification. Phosphoric acid and potassium hydroxide, used for adjusting pH of mobile phase, were purchased from BDH, UK. PAR is prepared as reference standard by methanolic extraction from PAR tablets (PARALIEF, Clon Medica, Irland) with m. p. 260 °C, CAF is obtained from PRS Panreac (Spain), MET from Riedel-de Haen (Germany) and ASC from ANALAR, UK.

Pharmaceutical formulation

Commercial pharmaceutical samples of Panadol extra tablets containing 500 mg PAR and 65 mg CAF (Teriak, Egypt), Hepamol tablets containing 500 mg PAR and 100 mg MET (Hikma,, Egypt), Efferalgan Vitamin C effervescent tablets containing 330 mg PAR and 220 mg ASC (UPSA,Tunisie) were purchased from the local market of EL-Beida city (Libya)

Apparatus

Chromatographic separation is performed on modular HPLC system PE-200 Perkin Elmer (USA) arranged with a P200 pump, solvent degasser DGU-3A, Rheodyne injector with 100 µL loop, automatic sampler AS200, Peltier Sample Tray 200 adjusted at 15 °C, UV200 UV detector with controlled wavelengths as detailed in table 1 and communication Network Chromatography Interface NCI 900. A Brownlee Bio C18, 250 mm x 4.6 mm, 5 µm particle size column is used as a stationary phase. The components are separated isocratically with a mobile phase consisting of water-acetonitrile (85:15 v/v) degassed under vacuum at a flow rate of 1.0 mL min^{-1} and temperature 15 °C.

This low operating temperature is used because the stability of ascorbic acid decreases with increasing temperature. The injection volume is 10 µL. The system is controlled and data analyses are performed with the TotalChrom Workstation Navigator software and peak areas are estimated by Microsoft ORIGIN software program (version 6). Linearity data are computed on a personal computer using Microsoft Excel program (version 2003, Microsoft Co., Redmond, USA).

Preparation of standard stock and calibration solutions

ForPAR, MET, ASC and CAF standard solutions**,** 50 mg each of standard powdered analytes were weighed, transferred to 4 separate 100 mL volumetric flasks, dissolved in distilled water and completed to the mark having final concentrations of 500 µg mL^{-1} each.

Series 10 dilutions of the standard stock solutions are made separately by pipetting out 0.25 up to 10 mL of standard stock solutions into separate 25 mL volumetric flasks and diluting to volume with distilled water to produce the concentrations ranging from 5-200 µg mL^{-1}.

Preparation of synthetic quaternary mixtures

Equal volumes (3 mL) of the working standard solutions of each drug are transferred into a 25-mL volumetric flask to prepare synthetic quaternary mixtures of PAR with MET, ASC or CAF. The solutions are then diluted with distilled water to the volume.

Preparation of pharmaceutical samples

One tablet is vigorously dissolved in distilled water with magnetic stirrer, transferred to 250 mL volumetric flask and completed to the mark with distilled water. The stock solution is filtered through a Whatman Filter paper number 42 and 1 mL of the filtrate is transferred to 25-mL volumetric flasks and diluted to the mark with distilled water.

Linearity and range

The linearity of the method is determined at six concentration levels ranging from 5 to 200 µg mL^{-1} for each component. The calibration curves are constructed by plotting peak heights versus concentrations of cited components, and the regression equations are calculated.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

During preliminary investigations of chromatographic behaviour of CAF, PAR, ASC and MET the influence of mobile phase composition at various ratio and its pH value were investigated. From the chromatograms shown in it is evident (Figure 1), that a mobile phase consisting of (water-acetonitrile) (85:15) (v/v) is most suited to achieve fast and maximum separation and sensitivity.

It is evident also that variation in pH value does not improve in the separation (Figure 2). The chromatographic run time is 6 min and the dead time t_0 is 0.85 min which is defined by the time required to move the mobile phase from the injection loop, capillary connections and the column to the detector as demonstrated in Figure 1.

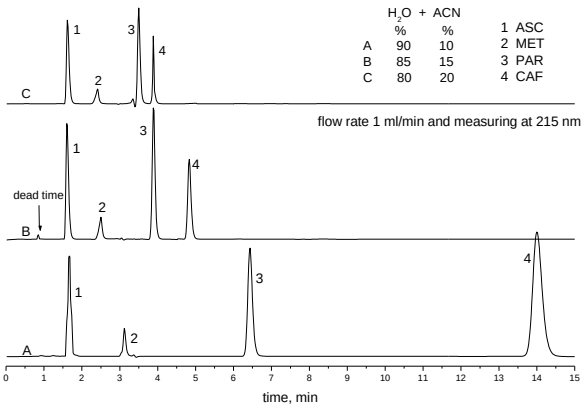

Figure 1. Effect of composition of mobile phase on the separation performance with flow rate 1 mL min^{-1} and monitoring at 215 nm.

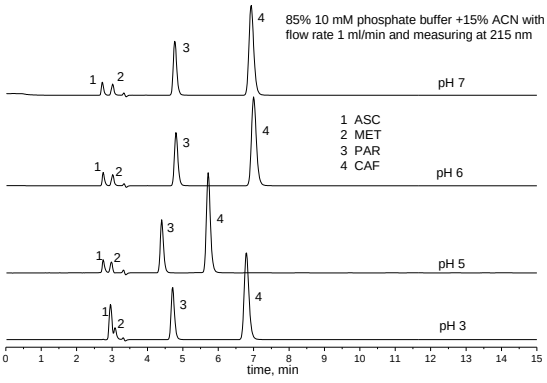

Figure 2. Effect of 10 mM phosphate buffer at different pH on the separation performance.

Based on the highest UV absorbance for ASC, MET, PAR and CAF, 260, 200, 240 and 270 nm are chosen for detection of this new HPLC method at which the best detector responses for all substances are obtained. The overlapped UV Spectrum is shown in Figure 3. The proposed method is subjected to validation for various parameters like system suitability, specificity, range and linearity, accuracy, precision and robustness in accordance with International Conference on Harmonization guidelines.

Linearity

Figures 4 through 7 demonstrate linearity of peak height with the concentration and Table 1 presents the slope and intercept of the regression line, correlation coefficient (*r*). Excellent linearity is obtained for all compounds between the peak heights and concentrations of 10-120 µg mL^{-1} with r = 0.9919, 10-400 µg mL^{-1} with r = 0.9976, 10-240 µg mL^{-1} with r=0.9954 and 10-240 µg mL^{-1} with r=0.9995 for ASC, MET, PAR and CAF, respectively.

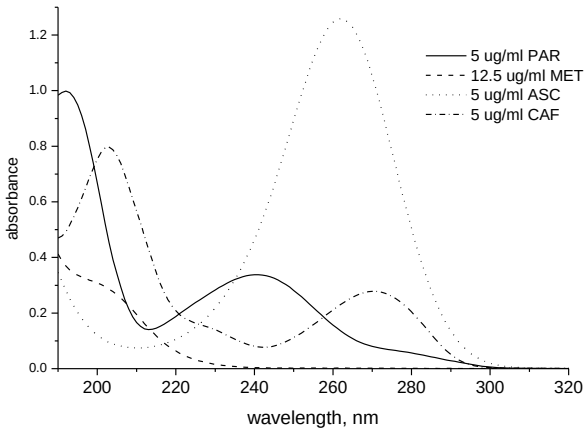

Figure 3. Overlapped spectra of ASC, MET, PAR and CAF.

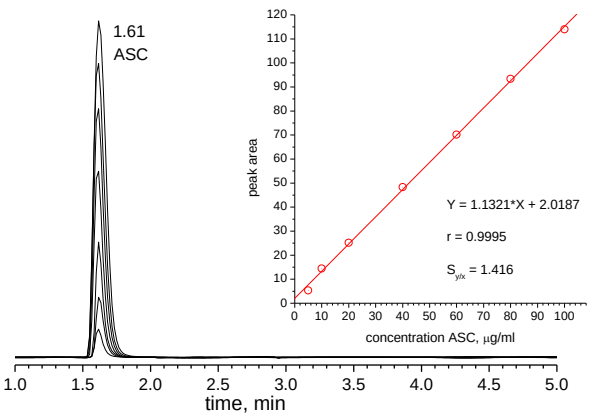

Figure 4. Chromatogram of ASC at different concentrations and its corresponding calibration curve.

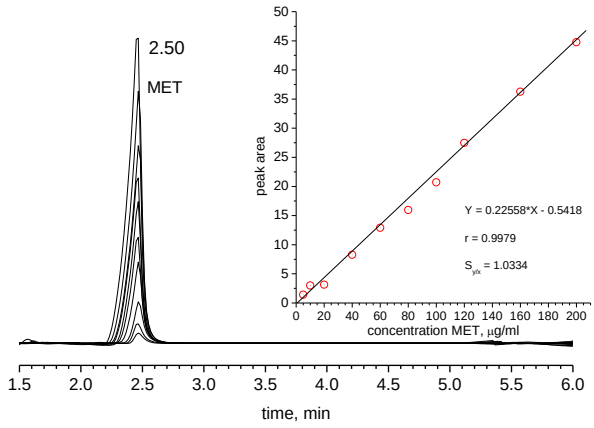

Figure 5. Chromatogram of MET at different concentrations and its corresponding calibration curve.

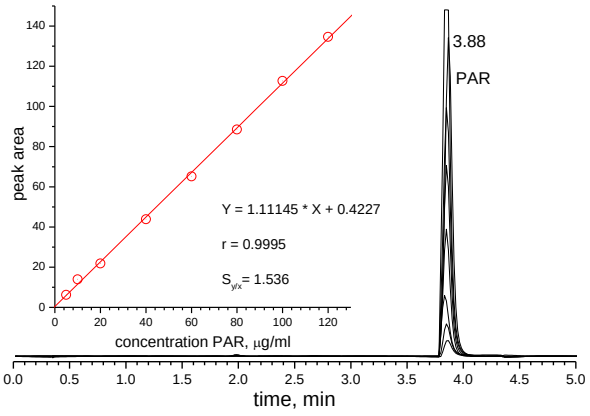

Figure 6. Chromatogram of PAR at different concentrations and its corresponding calibration curve.

Limits of detection and quantification

Sensitivity of the proposed method is estimated in terms of limit of detection (LOD) and limit of quantification (LOQ). LOD, which is defined as the lowest active substance concentration that can be determined by a method, usually cannot be calculated precisely and accurately. On the other hand, LOQ is the concentration of the sample used in analysis that can be obtained with adequate precision and accuracy. These limits are estimated by the two following equations.

$$LOD = \frac{3 \times S_{y/x}}{S} \quad (1)$$

$$LOQ = \frac{10 \times S_{y/x}}{S} \quad (2)$$

where $S_{y/x}$ is the residual standard deviation and S is the slope of the regression line.

The LOD is calculated to be 3.75, 13.74, 4.14 and 10.50 µg mL^{-1} and the LOQ is calculated to be 12.50, 45.81, 13.82 and 35.00 µg mL^{-1} for ASC, MET, PAR and CAF, respectively

Suitability of the method

The resolution, R_s, of two neighbouring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using Eqn. (3).

$$R_s = (t_{R1} - t_{R2})/0.5(t_{w1} + t_{w2}) \quad (3)$$

The calculated resolution values between each peak-pair are no less than 3.60 and the selectivity is not less than 1.30. Three fundamental parameters that influence the resolution of a chromatographic separation are capacity factor (*k*'), selectivity (α) and column efficiency (*N*). These parameters should be provided by different means to achieve better resolution, as well as defining different problem sources.

Capacity or retention factor (*k*') is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of *k*' ranges from 2-10. Capacity factor can be determined by using Eqn. (4).

$$k' = \frac{t_R - t_0}{t_0} \quad (4)$$

where t_R = retention volume at the apex of the peak (solute) and t_0 = void volume of the system or alternatively dead time.

Table 1. Linearity results, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	Range µg mL^{-1}	slope	Intercept	r	$S_{y/x}$	LOD µg mL^{-1}	LOQ µg mL^{-1}
ASC	5-100	1.1321	2.0187	0.9995	1.416	3.75	12.50
MET	5-200	0.2256	-0.5418	0.9979	1.033	13.74	45.81
PAR	5-120	1.1114	0.4227	0.9995	1.539	4.14	13.82
CAF	20-160	0.2427	4.7226	0.9978	0.8496	10.50	35.00

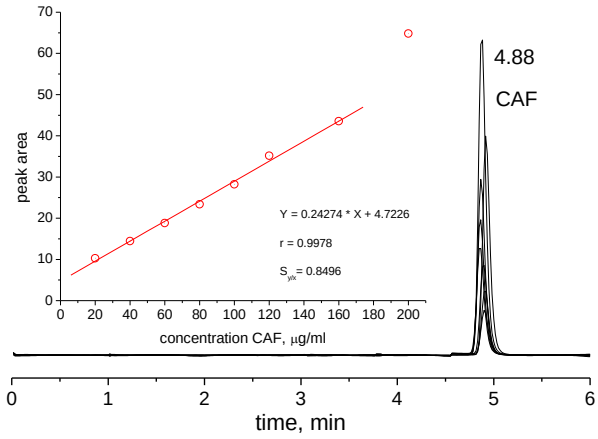

Figure 7. Chromatogram of CAF at different concentrations and its corresponding calibration curve.

Ideally, the retention factor for an analyte is between one and five. Lower values may give inadequate resolution. Higher values are usually associated with excessively brood peaks and unacceptably long run times. Increasing the retention factor is accompanied with decreasing the water content in mobile phase.

The s eparation or selectivity factor (α) is the ratio of the capacity factors of two adjacent peaks and represents the separation power of particular adsorbent to the mixture of these particular components. The selectivity factor is always greater than one and the ideal value is 2 providing species A elutes faster than species B. It can be calculated by using Eqn. (5).

$$\alpha = k'_B/k'_A \qquad (5)$$

Selectivity (α) value is sensitive to changes in pH, ionic strength or temperature.

Column efficiency (N) is a measure of number of theoretical plates per meter and is calculated by using eqn. (6).

$$N = 16\times\left(\frac{t_R}{w_b}\right)^2 \qquad (6)$$

where

N = number of theoretical plates,

t_R = elution volume, retention time or retention distance (mL, sec, or cm) and

w_b = width of the peak at the base line (mL, sec, or cm).

Alternative formula is given in Eqn. (7).

$$N = 5.54\times\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (7)$$

where

$w_{1/2}$ = width of the peak at half peak height (mL, sec, or cm).

Columns with N ranging from 5,000 to 100,000 plates meter^{-1} are ideal for a good column efficiency. The column is degraded when the peak is broaden and to make sharp peak, increase number of plates in the column either by decreasing flow rate, decreasing injection volume or diluting the sample.

Peak asymmetry or tailing factor T is a measure of column performance. At 10 % peak height, the asymmetry factor is given by Eqn. (8).

$$A_s = ba \qquad (8)$$

where

A_s = peak asymmetry factor,

b = distance from the point at peak midpoint to the trailing edge (measured at 10 % of peak height) and

a = distance from the leading edge of peak to the midpoint (measured at 10 % of peak height).

At 5% peak height, the tailing factor is given by Eqn. (9).

$$T = \frac{a+b}{2\times a} \qquad (9)$$

where

T = tailing factor (measured at 5% of peak height),

b = distance from the point at peak midpoint to the trailing edge and

a = distance from the leading edge of the peak to the midpoint.

A_s values of 1.00-1.05 are taken as excellent, those between 1.05-1.20 as acceptable. The A_s value of 2 and above are considered unacceptable. For a well packed column, a tailing factor of 0.9 to 1.2 should be achievable.

From the chromatogram shown in figures 1 and 2, it is evident, that under the proposed chromatographic conditions, ASC, MET, PAR and CAF are completely separated, which is indicated that the method is selective and could be used for their simultaneous identification and quantification. System suitability test is an integral part of chromatographic methods and is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed.

Table 4. Recovery Results for ASC, MET, PAR and CAF in Synthetic Mixtures by RP-HPLC

Mixture No.	ASC		MET		PAR		CAF	
	taken	%	taken	%	taken	%	taken	%
1	60	98.56	60	101.54	60	98.62	60	101.47
2	120	102.70	60	96.33	60	101.04	60	95.82
3	60	96.46	120	97.41	60	102.56	60	99.36
4	60	100.94	60	99.20	120	98.31	60	102.13
5	60	97.08	60	103.43	60	102.73	120	97.44
Mean, %		99.15		99.58		100.65		99.24
RSD, %		2.65		2.93		2.09		2.68

The chromatographic parameters of resolution R_s, retention factor k', selectivity α and peak asymmetry T are satisfactory for these compounds as given in table 2.

Table 2. System suitability parameters of ASC, MET, PAR and CAF.

Compound	t_R	N	k'	T	α	R_s
ASC	1.62±0.025	3297	0.90	1.15		
					2.11	5.27
MET	2.47±0.058	3875	1.90	1.11		
					1.86	8.33
PAR	3.83±0.008	17651	3.53	1.32		
					0.91	6.83
CAF	4.89±0.014	19059	3.42	0.64		

Flow rates between 0.5 and 1.5 mL min^{-1} were studied. A flow rate of 1.0 mL min^{-1} gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed-phase C18 column, the retention times for ASC, MET, PAR and CAF are observed to be 2.88, 3.22, 4.29 and 4.93 min respectively. Total time of analysis was less than 6 min.

Precision and accuracy

A standard working solution containing ASC, MET, PAR and CAF, yielding final concentrations of 60 µg mL^{-1} for each is prepared and is injected 5 times as a test sample. From the calibration curves of each compound, the concentrations of the ASC, MET, PAR and CAF are calculated using the detector responses. The precision of the method, expressed as the RSD % is 3.99, 4.95, 1.44 and 5.63% for ASC, MET, PAR and CAF, respectively. The accuracy, defined in terms of % deviation of the calculated concentrations from the actual concentrations, is listed in table

Analysis of synthetic mixtures

With the calibration curve of the corresponding standard component, calculate the concentration (in milligram per liter or microgram per milliliter) of the analyte in the test solution by using eqn. (10).

$$[\text{Analyte}] = \frac{(A-I)}{S} \quad (10)$$

where

A = the peak area of the analyte in the test solution,

I = the y-intercept of the 5-point calibration curve,

s = the slope of the 5-point calibration curve.

Recovery studies in this method are performed on the synthetic mixtures prepared by adding accurately weighed amounts of the drugs Mean recoveries and RSD are found to be 101.62 and 4.28% for ASC, 101.62 and 4.28% for MET, 100.11 and 3.38% for PAR 100.86 and 2.88% for CAF, respectively.

Table 3. Precision and accuracy of the Developed Method (n= 5).

Compound	Added µg mL^{-1}	Found µg mL^{-1}	RSD %	Deviation %
ASC	60	62.28 ± 2.63	3.996	-3.80
MET	60	54.43 ± 2.49	4.953	5.95
PAR	60	57.94 ± 0.87	1.444	3.43
CAF	60	63.11 ± 3.38	5.630	-5.18

Application of pharmaceutical formulations

Assay results for the determination of PAR with MET, ASC or CAF in commercial pharmaceutical are given in tab RSD (%) indicates the accuracy of determination of active ingredients in the investigated pharmaceutical preparations.

Conclusion

The developed method is suitable for the identification and quantification of the quaternary combination of ascorbic acid, methionine, paracetamol and caffeine. A high percentage of recovery shows that the method can be successfully used on a routine basis. The proposed method is simple, sensitive, rapid, specific and could be applied for quality and stability monitoring of PAR with either ASC, MET or CAF combinations.

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Received: 14.06.2017.
Accepted: 20.07.2017.