UJPAH **Vol. I** **No. 18** **June 2015**

Abstracted & Indexed by CAS, a division of American Chemical Society

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Contents

EDITORIAL

India urgently needs specific measures for the development of Pharmaceuticals from all quarters. The recently passed Budget for 2015-16 has no incentives on R&D and investments in Pharma or any significant tax incentives for export and innovation, therefore, it does not enable the culture of "India innovates" to encourage young scientists and give them a line to focus on innovation. Even the current Budget does not reverse the service tax imposed on Clinical research organizations introduced last year. Anyhow whatever may be the political thinking, the young scientists and non political organizations follow their respective duties towards the development of Indian Pharma Industry through innovation and documentation of the therapeutic effects of Indian herbs. UJPAH is also committed and have not received/took any financial assistance/monetary help for the services UJPAH is rendering since its inception in the year 2005.

I am happy about the relationship I have in person with the Members of the Board of UJPAH where each one of them voluntarily is supportive for the cause of encouraging the young Scientists working on Indian herbs.

Similarly, I congratulate the young scientists of the Universities, their departmental Heads and other officials who deserve gratitude for the support rendered in our endeavour by sending students and staff members for making this mission successful.

In the end, my special thanks to Dr. Rajendra Dobhal, DG-UCOST who always stood for the cause like my younger brother.

(Dr. S. Farooq)
Chief Editor

TlC and Spectroscopic Based Determination of Free Radical Scavenging Activity of Seven Ayurvedic Medicinal Plants

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Abstract- Antioxidant activity of medicinal plants is widely recognized for their therapeutic value. Various *in-vitro* and *in-vivo* methods are used to assess antioxidants in plant extracts. However, in the screening of large number of plants, rapid method of detection of active compounds is required to develop further activity guided fractionation of the extracts. In this study, methanolic crude extracts of seven plants namely *Balsamodendron mukul*, *Boerhavia diffusa, Catharanthus roseus, Curcuma longa, Lawsonia inermis, Laurus nobilis* and *Piper nigrum* were subjected to phytochemical analysis by color test and TLC for detection of phytocompounds, particularly flavonoids. DPPH bio-autographic method was used to detect the antioxidant activity of particular spot on the pre developed TLC plate. Further each extract was also assessed by standard spectroscopic analysis for DPPH radical scavenging potential. The data obtained is comparable and highlights the importance of TLC based method for fast detection of antioxidant active phytocompounds in the crude extract of the plant/Ayurvedic drugs.

Key words: Antioxidant activity, Medicinal plant, Phytocompound, TLC.

Introduction

Free radical facilitated cell damage plays an important role in the development of numerous human diseases. It has been widely assumed that progression of diseases such as cardiovascular disease, neuronal disease, cataracts, and several types of cancer are mediated with free radicals generation that ultimately leads to the onset of oxidative stress (Thomas & Kalyanaraman, 1997). Recent clinical studies have indicated positive role of natural antioxidant in significant reduction in the risk of coronary heart disease and ageing related disease (Valko et al., 2007). On the other hand, certain toxicity potential and carcinogenicity of synthetic antioxidants such as BHA and BHT have been reported (Sasaki et al., 2002). Therefore, there is an increasing interest in search of natural antioxidant from plants such as vegetables, fruits and medicinal herbs. Antioxidant activity in plants is very commonly reported but it is challenging to rapidly screen the most active antioxidants in the plant extracts due to its complex nature and presence of large number of interfering constituents. Numerous methods have been developed for screening of plant extracts for their antioxidant potential, among these TLC-bio-autography method (Cimpoiu, 2006) can quickly detect and separate the antioxidant active constituents from complex crude extracts (Gu et al., 2009).

Various plants used in the traditional medicinal system produce a lot of antioxidants and represent rich source of new and comparably nontoxic phytocompounds with antioxidant activity. It has been widely accepted that antioxidant active secondary metabolites including simple phenolic acids to highly polymerized tannins impart therapeutic potential to various plants (Pandey and Rizvi, 2009). Moreover, majority of plants derived antioxidant active compounds falls in the category of polyphenolics (e.g. phenolic acids, flavonoids, and tannins) and among these flavonoids represent most versatile group in terms of their antioxidant and other therapeutic values (Cimpoiu, 2006). Depending upon the chemical modification in the basic benzo-γ- pyrane ring structure, flavonoids chemically subdivided into flavonols, flavones, isoflavones, flavanones and anthocyanidins (Harborne and Baxter, 1993). Characterization of these biologically active phytochemicals present in the plant along with the

knowledge of their antioxidant potential can give fair estimate of their therapeutic potential (Chanda et al., 2011). Furthermore, simultaneous separation and detection of phtycompounds with antioxidant activity from crude mixture of plant extracts can ultimately lead to identification of most potent antioxidant molecules.

In our previous studies, a large number of Indian medicinal plants were screened for their broad spectrum antioxidant activity and also identified the active fractions/compounds (Ahmad et al., 2008; Zahin et al., 2009, 2010, 2013). In the present study, DPPH-TLC bio-autography of crude extract of seven traditionally used medicinal plants namely *B. mukul*, *B. diffusa*, *C. roseus*, *C. longa*, *L. inermis*, *L. nobilis* and *P. nigrum* was performed along with spectroscopic analysis for DPPH free radical scavenging activity. In addition, TLC based detection of flavonoids and phytochemicals profiling was also carried out using standard protocol.

Material and Methods

Collection of essential oil samples: The authentic plant materials were collected in the vicinity of University campus (AMU) or obtained from local authorized shops. The identification of the samples was further confirmed by the Department of Botany, Aligarh Muslim University, Aligarh, India. The voucher specimens have been deposited in the Department of Agricultural Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh. The detail of the plant samples along with their parts used has been summarized in table 1.

Preparation of plant extract: Plants extract was prepared as described earlier (Ahmad and Beg, 2001) with a little modification. The collected plants-material was dried in the shade. 100 gm of ground plants material was soaked in 300ml of methanol for 3-4 days and stirred with glass rod after every 18 hrs. Plants-material was filtered using Whattman filter paper No.1. The filtrate was concentrated in a rotatory evaporator at 40° C temperature under vacuum. All extracts were stored at 4° C for further use.

Phytochemical analysis of plant extracts: Plants extract were screened for the presence of major phytocompounds by colour test using standard method as describe by Jamil et al.(2012).

TLC based detection of Flavonoids in plant extracts: TLC was performed on silica gel 60 F_{254} plates (Merck, Germany) using a solvent system of n-hexane–toluene–ethyl acetate–formic acid (2:5:2.5:0.5) as described by Wagner and Bladt (1996). Aliquot of plant extracts-methanolic solution (1 mg/mL) was directly spotted onto the TLC plates for development, followed by spraying with Natural product-polyethylene glycol reagent. Typical intense fluorescence indicates different classes of flavonoids as follows:

Flavonols: Orange-Yellow Fluorescence, Yellow-green Fluorescence

Flavones: Orange Fluorescence, Yellow-green Fluorescence

Phenol carboxylic acid: Blue Fluorescence

TLC-DPPH bio-autography analysis: An aliquot of plant-extract methanolic solution of concentration 1mg/mL, was directly applied (as spot) using Pasteur pipette onto the TLC plates (Silica gel 60 F_{254}). TLC plates were developed in a presaturated solvent chamber with n-hexane–toluene–ethyl acetate–formic acid (2:5:2.5:0.5) as developing reagents. The developed TLC plates were then removed from the chamber sprayed with a 1 mM DPPH radical methanol solution for derivatization. Bands with the DPPH radical scavenging activity were observed as white yellow bands on a purple background (Gu et al., 2009).

Total phenolic compound analysis: The amount of total phenolic in plant extract was determined with the Folin-Ciocalteu reagent using the method of Spanos and Wrolstad (1990), as modified by Lister and Wilson (2001). To 5 ml of each sample (three replicates), 2.5 ml 1/10 dilution of Folin-Ciocalteau's reagent and 2 ml of Na_2CO_3 (7.5% w/v) were added and incubated at 45°C 15 min. The absorbance of all samples was measured at 765 nm using a Spectronic 20D+ UV–Vis spectrophotometer. Result was expressed as milligrams of gallic acid equivalent per dry weight of plant extract (mg GAE/g dw).

Estimation of flavonoids content: The flavonoids content in extracts was determined

spectrophotometrically according to Lamaison and Carnat as describe by Quettier-Deleu et al. (2000), using a method based on the formation of a complex flavonoid–aluminium, having the absorbtivity maximum at 430 nm. Rutin was used as control to make the calibration curve. 1 ml of diluted sample was separately mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with a Spectronic 20D+ UV–Vis spectrophotometer and the flavonoids content was expressed in mg per g of rutin equivalent (RE).

Free radical scavenging assay: The scavenging activity of DPPH free radicals by different plants-extracts was assayed according to the method described by Gyamfi et al., (1999). Fifty micro liters of the plants-extract in methanol, yielding 12.5, 25, 50 and 100µg extract respectively in each reaction was mixed with 1ml of 0.1 mM DPPH in methanol solution and 450µl of 50mM Tris-HCL buffer (pH 7.4). Methanol (50µl) only was used as control of experiment. After 30min of incubation at room temperature the reduction of the DPPH free radial was measured at 517nm. Vitamin C was used as controls. The percent inhibition was calculated from the following equation:

% Inhibition = [Absorbance of control – Absorbance of test sample / Absorbance of control] X 100

Results

Phytochemical Analysis: Preliminary phytochemical analysis of methanolic crude extract of seven selected plant extracts revealed the presence of Alkaloids, flavonoids, tannins, glycosides, and saponins in one or more plant extracts as summarized in Table-1.

TLC analysis of plant extract for detection of Flavonoids: Different classes of flavonoids were detected in the methanolic crude extract. All the plant extract showed varying degree fluorescence quenching band at UV-254 nm that showed the possible presence of flavonoids and phenolic acids (fig. 1A). Typical intense fluorescence in UV-365 nm is produced immediately after spraying the natural product reagent (fig. 1 B) which confirmed the presence of flavonoids in the plant-extract. Based on the fluorescence type, the presence of flavanols, flavanones and phenol carboxylic acid were characterized in each extract as major compounds. Number and antioxidant activity of flavonoid bands observed in each plant extract varied are shown in fig. 3.

Calorimetric estimation of Total Phenolics and flavonoids : The amount of total phenol was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent. The results for total phenolic and flavonoid content in crude-methanolic extracts are presented in table-1. Total phenolic contents were significantly high in the crude extract of *B. diffusa* (589.92 mg GAE $10g^{-1}$) and least was in *L. nobilis* (210.34 mg GAE $10g^{-1}$). The amount of phenolic compound ranged from 702.34 to 210.34 mg GAE g^{-1}dry weight of all the medicinal plants examined. The contents of total Falvonoid that were measured by $AlCl_3$ reagent in terms of Rutin equivalent are shown in table-1. Flavonoid content of extracts varied from 32.51 (*L. nobilis*) to 95.38 (*L. inermis*) mg Ru $10g^{-1}$. The results of table show that the total phenolic content is higher in *B. diffusa*, whereas the flavonoids presence is most significance in *L. inermis*.

TLC based detection of antioxidant activity and Free radical scavenging assay: In the present study, methanolic plant-extracts are monitored by a TLC bio-autography method because this method gives quick access for detection and localization of the active compounds in a complicated plant extract (Tasdemir et al., 2004). In the method, the DPPH scavenging activity was observed visually as white yellow spots on a purple background. Fig.1C shows a profile of the antioxidant components in methanolic extract of *B. mukul*, *B. diffusa*, *C. roseus*, *C. longa*, *L. inermis*, *L. nobilis* and *P. nigrum* under visible light. The chromatograms of all the plant-extracts were observed to have DPPH scavenging activities. The entire band that were previously detected as flavonoids (fig. 1B), observed to have radical scavenging activity (fig. 3). Our findings of TLC bioautography are in agreement with the reports of Cimpoiu (2006) who has detected such activity of flavonoids by TLC. However, DPPH-bio-

autography of different plant-extracts also revealed that bands other than of flavonoid, also exhibit potent antioxidant activity.

DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid was used as standard. The extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. The scavenging activity of the seven extracts tested was compared to those of ascorbic acid, used as positive control, and was found to be concentration-dependent. This activity can be evaluated by the determination of the IC_{50} values, corresponding to the amount of extract required to scavenge 50% of DPPH radicals present in the reaction mixture. High IC_{50} values indicate low antioxidant activity. As shown in Table-1, the scavenging effect in terms of IC_{50} value of methanolic extracts and standards radical is in the following order: *B. mukul* (47.61 µg/ml) > *B. diffusa* (49.53 µg/ml) > *L. inermis* (52.02 µg/ml) > *L. nobilis* (53.98 µg/ml)> *C. longa* (55.28 µg/ml)> *C. roseus* (60.26 µg/ml) > *P. nigrum* (62.61 µg/ml), control compound (ascorbic acid) shows the IC_{50} value of 8.26 µg/ml. From fig. 2, a dose dependent response was found in the DPPH radical scavenging activity; the activity increased as the concentration increased for each individual crude methanolic extracts.

Our findings are in accordance with the report of other workers (Hsouna et al, 2011; Shisode and Kareppa 2011; Tiong et al, 2013; Shabana and Afifi, 2014; Khan et al., 2014). Free radical scavenging and antioxidant activity of the phenolics/flavonoids mainly attributed to the numbers and position of hydrogen donating hydroxyl groups on the aromatic ring of the phenolic molecules, and also affected by others factors such as glycosylation of aglycones, other H-donating groups (-NH, -SH), and so on. Substitution patterns in the B-ring and A-ring as well as the 2,3 double bond (unsaturation) and the 4-oxo group in the C-ring also affect antioxidant activity of phenolics (Cai et al., 2006). As a result, of their abilities to scavenge free radicals, phenolics exhibited excellent antioxidant, antimutagenic, antiinflammatory, and anticancer properties (Ahmad and Mukhtar 1999). Therefore, therapeutic properties of the plants tested may possibly be attributed to the presence of phenolic compounds in particular, the flavonoids present in the plant extracts.

Conclusion

On the basis of this preliminary study, it is concluded that TLC based detection and identification of antioxidant compounds is a simple and fast method for screening of large number of extracts. Antioxidant potency of the extracts by spectroscopic method revealed similar trend. Further, the TLC based approach may be developed into more efficient technique using HPTLC for fast detection and quality assurance of herbal extracts and isolation of most active antioxidant.

Acknowledgement

We are grateful to Dr. S. Farooq (Director, The Himalaya Drug Company, Dehradun) for his guidance and support in above work.

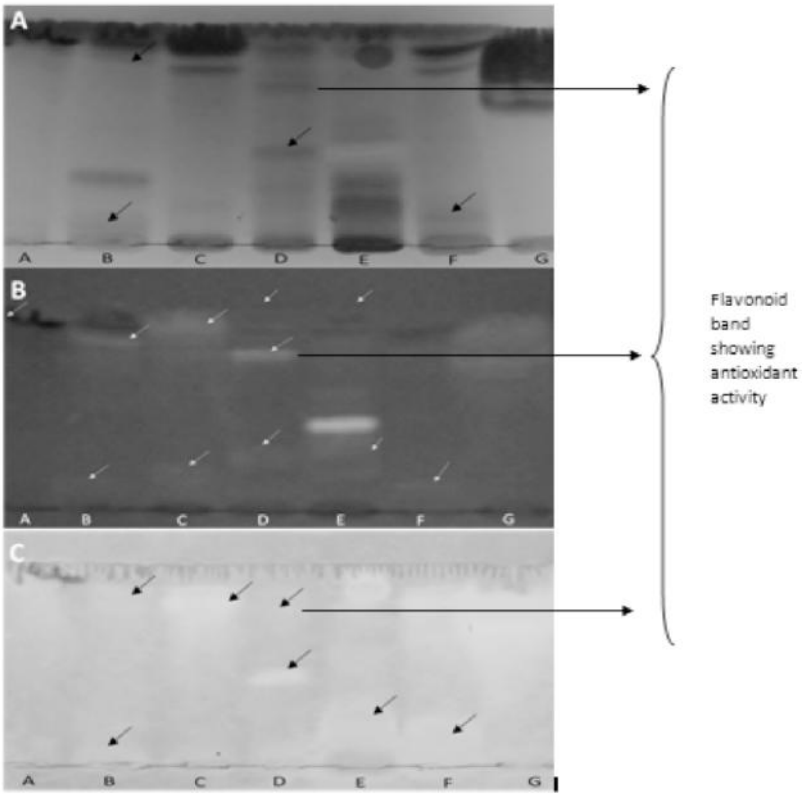

Fig.1 TLC analysis of plant extracts A) shows chromatogram of plant extracts at 254 nm. B) tlc chromatogram showing the detection of flavonoids corresponding to different classes at 366nm and C) DPPH-TLC bioautography showing different antioxidant active bands. Plant extracts spot lane: A) *Curcuma longa*, B) *Piper nigrum*, C) *Lawsonia inermis*, D) *Boerhavia diffusa*, E) *Catharanthus roseus*, F) *Laurus nobilis* and G) *Balsamodendron mukul*

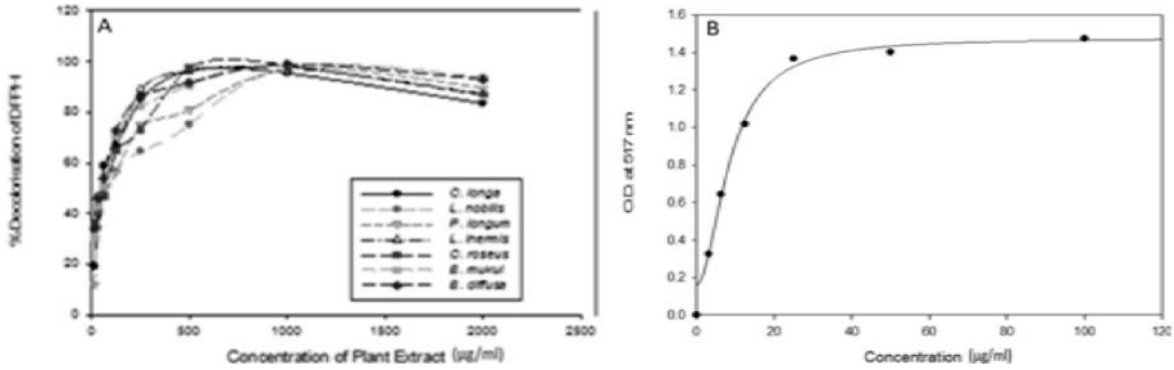

Fig. 2 (A) Free radicle inhibition activity of plant extracts and (B) standard curve of Ascorbic Acid.

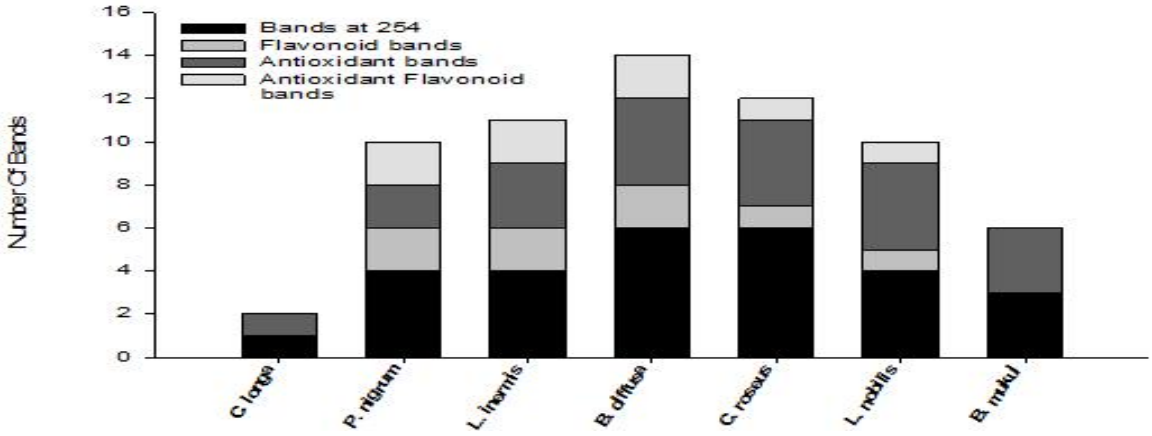

Fig. 3 Band profile of different plant extracts as observed on their respective chromatograms.

Table-1 Phytochemical analysis and antioxidant activity of methanolic extracts of Ayurvedic plants.

Plant name	Family	Part used	Phytochemical analysis					Total Phenolic content (mg GAE/10 gm)	Total flavonoid content (mg RU/10gm)	DPPH free radical scavenging activity (IC_{50} µg/ml)
			Alkaloids	Flavonoids	Glycosides	Tannins	Saponins			
Balsamodendron mukul	Burseraceae	Resins	+	+	+	+	-	258.34	80.65	47.61
Boerhavia diffusa	Nyctaginaceae	Root	-	+	+	-	+	589.92	58.81	49.53
Catharanthus roseus	Apocynaceae	Leaves	+	+	-	+	+	218.19	42.25	60.26
Curcuma longa	Zingiberaceae	Rhizome	+	+	-	-	-	347.94	60.72	55.28
Lawsonia inermis	Lythraceae	Leaves	+	+	+	-	-	368.70	95.25	52.02
Laurus nobilis	Lauraceae	Leaves	-	+	-	-	+	210.34	32.51	53.98
Piper nigrum	Piperaceae	Fruit	+	+	+	-	-	283.68	35.58	62.61

The data represents mean values of three independent experiments.

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Chemical Constituents From Bark of *Euonymus tingen* (Celastraceae)

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Abstract- 1,5 di-hydroxy 3,6,7 tri methoxy 8-allyloxy xanthone and 5-O-α-L xylopyranosyl (1→6) β-D-glucopyranosyl 1,6 dihydroxy 3,5 dimethoxy xanthone were isolated from bark of *Euonymus tingen*. The structures of these compounds were characterized by means of chemical and spectral methods including advanced 2D NMR studies. These compounds were first time isolated from this species.

Key words: *Euonymus tingen* Celastraceae, 1,5-di-hydroxy 3,6,7 tri methoxy 8-allyloxy xanthone, 5-O-α-L Xylopyranosyl (1→6) β-D-glucopyranosyl 1,6 dihydroxy 3,5 dimethoxy xanthone.

Introduction

Euonymus tingens (Roxb.), vern. Kumkum, family Celastraceae, is a evergreen tree or large shrubs, often attaining to 8m high; bark dark ash-coloured, bright yellow inside. Leaves opposite, elliptic or ovate-lanceolate. Flowers pale-white, calyx lobes fimbriate. Petals orbicular, with yellowish tingen; stemns shorter than petals; seeds dark brown, half enclosed in orange-red aril. Flowering season is March to April and fruiting from May to September. The plant is distributed from W. Himalaya, Shimla to Bhutan; Myanmar and Tibet[1]. The bark paste of the plant is useful in eye diseases. Number of Tingenone, hydroxytingenone, triterpenoid quinine methides has been isolated from *Euonymus tingen*[2,3,4].

Material and Methods

Plant material- Stem bark (6 kg) of *Euonymus tingen* was collected from Moling Ghat, District Chamoli Garhwal during October 2014 and identified by taxonomist in the Department of Botany, H.N.B. Garhwal University Srinagar. A voucher specimen (GUH-8327) of the plant is deposited at the Herbarium of Department.

Extraction and isolation- Shade dried and coarsely powdered stem bark of *Euonymus tingen* (4 kg) was extracted thrice with 95% ethanol (5L) at 50°C (15 hrs) on a heating mantle. The reaction mixture was filtered off and the filtrate was concentrated under reduced pressure to yield brownish residue (440 g). This residue was fractionated with EtOAc (repeatedly 3-4 times) yield EtOAc soluble and insoluble fraction. EtOAc soluble portion (310 g) with n-hexane: Chloroform (93:7) as eluting solvent with increasing polarity of $CHCl_3$, afforded two compounds in pure form tentatively designed as compound **1** and **2** respectively. These compounds were purified by recrystallization.

General experimental procedure- UV spectra were taken in MeOH using $AlCl_3$ as shift reagent. IR recorded in KBr on a Perkin Elmer FT IR Spectrophotometer. ^{1}H-NMR were run at 300MHz using TMS as internal standard and C_5D_5N and DMSO as solvent. ^{13}C-NMR recorded in 125MHz, using C_5D_5N and DMSO as solvent, FAB-MS on JOEL. JMS700 Mstaion Spectrophotometer. MPs were incorrected.

Result and Discussion

Compound 1

It was crystallized from methanol as yellow sticky mass.

Melting Point : 322-325 °C

Molecular Formula : $C_{21}H_{22}O_8$

U.V.(λ_{max}^{MeOH}) nm : 269,260

I.R.(ν_{max}^{KBr}) cm^{-1} : 1640

FAB-MS (*m/z*) : 402[M]$^+$, 371[M-OCH_3], 340[M-2xOCH_3],292[M-3xOCH_3+OH], 283[M2xOCH3+2xOH],199[M-3xOCH_3+2xOH+C_5H_8O]

^{1}H-NMR($CHCl_3$,¢ppm) : 1.65(s), 1.78(s), 2.57(s) 3.2(s), 3.44(s), 3.9(d,J=7.4Hz), 6.93 (d,J=8.6Hz), 7.6(d, J=7.4Hz), 9.8(s), 12.4(s,-OH).

^{13}C-NMR ($CHCl_3$, ¢=ppm)

Carbon No. (aglycone)	Chemical Shift (δppm)	Carbon No. (aglycone)	Chemical Shift (δppm)
C-1	161.6	C-9b	103.8
C-2	99.0	C-10	147.4
C-3	164.6	$-OCH_3$	56.5
C-4	94.2	$-OCH_3$	61.3
C-5	145.8	$-OCH_3$	60.4
C-6	148.5	$-CH_3$	15.4
C-7	148.2	$-CH_3$	11.82
C-8	160	CH=CH	122.6
C-9	176.6	CH=CH	122.8
C-9a	112.7	Tert-C	29.0

It was crystallized from MeOH, yellow sticky mass. It gave characteristic greenish brown colour with methanolic $Fecl_3$ and also positive to Gibbs test. Its positive FAB-MS spectra showed a molecular ion peak at *m/z* 402$[M]^+$, which correspond to the molecular formula $C_{21}H_{22}O_8$. Its UV spectrum in methanol showed a characteristic band at 269 nm and IR spectrum showed a bands at 3260 and 1640 cm^1 were characteristic for xanthone[5]. ^{1}H-NM of compound showed presence of two AB type doublets at δ 6.89(d,J=8.6Hz) and 7.5(d,J=7.4Hz) suggesting the presence of tetra substituted aromatic ring in the compound. The downfield signal at δ 12.4(s) and δ 9.8(s) exchangeable with D_2O indicate the presence of chelated hydroxyl group at C-1 position and one free phenolic OH group. The UV spectrum of MH-6 showed absence of $AlCl_3$ induced shift there by suggesting a bulky substituent located ortho to the hydrogen bonded group. Besides the $[M]^+$ ion peak, the FAB-MS of compound showed other significant peaks at m/z 340, 309, 292, 283 and 199 which correspond to the subsequent loss of three OCH_3, two OH and one C_5H_8O ion from molecular ion peak. The presence of dimethyl allyl group was further confirmed by ^{1}H-NMR spectrum of compound, which showed two methyl signals at δ 1.65(s) and δ 1.78(s), one 2H proton doublet at δ 3.9(d,J=7.4Hz) and a doublet at δ 7.5(d, J=7.4Hz). The peak at δ 6.89(d,j=8.6Hz) and δ 7.45(d, J=7.4Hz) showed the presence of three aromatic protons in ring A. The presence of three singlets at δ 2.57, 3.44 and 3.20 confirms the presence of three methoxyl group in compound.

^{13}C-NMR spectrum of compound showed the presence of twenty carbon signals. The downfield signal at δ 176.7 was attributed to α–β-unsaturated carbonyl carbon atom of xanthone[6]. The other signal at δ 56.7, 61.3 and 60.4 were assigned for methoxyl groups. The position of methoxyl groups were found at C-3, C-6 and C-7 atoms (by comparison of ^{13}C-NMR data with related compound[7]. The ^{13}C-NMR peaks at δ 11.82 and 15.14 showed the presence of two tertiary methyl groups, whereas the downfield peaks at 122.6 and 122.8 were compatible with a double bonded carbon. Two methyl signals at δ 1.65 and 1.78 9 (^{1}H-NMR) and tertiary carbon signal at δ 29.0 denoted the presence of gem-di methyl group. The carbon resonances at δ 161.6, 164.7, 148.2, 148.5, 145.9 and 160.0 showed the presence of six oxygenated carbon atoms and were compatible with 1, 3, 5, 6, 7, and 8 oxygenated carbon atoms of xanthone. Thus all these values were in agreement with reported data of 3, 3-di methyl allyl 3-methyl 2-hydroxy substituted xanthone. Hence on the basis of above spectral studies compound 1 was identified as **1,5-di-hydroxy 3,6,7 tri-methoxy 8-allyloxy xanthone** (Figure-1).

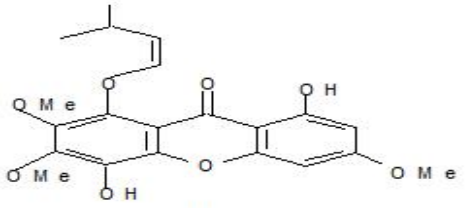

Figure -1

Compound 2

It was crystallized as yellow solid from methanol.

Melting Point : 185-187 °C

Molecular Formula : $C_{26}H_{30}O_{15}$

Molecular Weight : 582 amu

U.V.(λ_{max}^{MeOH})nm : 360,306,239,205.

I.R (ν_{max}^{KBr}) cm^{-1} : 3541(OH), 2926(C-H stretching), 1664(C=O), 1589(C=C stretching)

FAB-MS (*m/z*) : 621 [M + K]$^+$, 605[M+Na]$^+$, 583[M+H]$^+$, 503, 460.

^{1}H-NMR (C_5D_5N,¢-ppm)

3.82(3H,s,CH_3), 4.08(3H,s,CH_3), 13.10(s,OH), 4.33-4.40(-CH), 5.42(d,J=7.6Hz), 7.23(d,J=8.7Hz) 7.53(d,J=8.7Hz), 6.63(d, J=2.4Hz), 7.21(d,J=2.4Hz), 4.36,4.84(CH_2), 4.95(1H,d,J=7.3Hz), 4.04 (1H,dd, J=7.6,8.8Hz), 4.13(1H,t), 3.67(1H, d, J=10.2,11.2Hz).

^{13}C-NMR (C_5D_5N,¢-ppm)

Aglycone

160.7(C-1), 102.0(C-2), 165.0(C-3), 95.9(C-4), 158.6(C-4a), 149.7(C-4b), 146.5(C-5), 148.3(C-6), 123.4(C-7) 113.2(C-8), 118.3(C-8a), 176.2(C-9), 108.8(C-9a), 56.0 (OCH_3), 61.5(OCH_3).

Glycone

Glucose : 105.8 (C-1'), 75.1(C-2'), 77.6(C-3'), 71.4, (C-4'), 77.8(C-5'), 70.4(C-6')

Xylose : 106.6(C-1''), 75.0(C-2''), 78.2(C-3''), 71.1(C-4''), 67.2(C-5'')

It was crystallized from methanol as yellow solid. The FAB-MS of compound showed the molecular ion peak at *m/z* 621[M+K]$^+$, 605[M+Na]$^+$ and 583[M+H]$^+$ which correspond the molecular formula $C_{26}H_{30}O_{15}$ and molecular weight 582. Its UV spectrum showed characteristic bands at 360, 306, 239 and 206, suggesting a xanthone skeleton of compound. The IR spectrum displayed a bands at 3541 cm^{-1} (OH), 1664 cm^{-1} (C=O) and 1620 cm-1, 1589 cm^{-1} for (C=C) stretching. The ^{1}H-NMR spectrum showed a pair of ortho coupled protons which appeared at δ 7.53(1H.d,J=8.7Hz) and 7.23 (1H,d,J=8.7Hz) were assigned to H-7 and H-8, while two doublets of meta coupled protons at δ 7.21(1H,d,J=2.4Hz) and 6.63(1H,d,J=2.4Hz) were assigned to H-2 and H-4 respectively. Its ^{1}H-NMR spectrum also showed two separate singlet each integrating for 3H appeared at δ 3.82 and 4.06 were attributed for two methoxyl groups, attached to C-3 and C-5 carbon atoms.

The ^{1}H-NMR spectrum also displayed two sharp doublets at δ 5.42 (d,J=7.6Hz) and 4.95(d,J=7.3Hz) suggesting the presence of two sugar moieties in the compound, and were assigned for anomeric protons of glucose (H-1') and xylose (H-1''), while other protons of glucose moieties resonated between δ 4.22-4.82 and that of xylose at δ 3.67-4.35. The ^{13}C-NMR spectrum also supported that C-1' and C-2'' carbon atoms appeared at δ 105.8 and 106.6. Its ^{13}C-NMR spectrum further support the presence of twenty six carbon atoms. A signal for one proton appeared at δ 13.20 was assigned for chelated hydroxyl group. A downfield signal at δ 176.2 in ^{13}C-NMR spectrum attributed for α–β–unsaturated carbonyl group, which also confirms the xanthone skeleton of the compound 2 .

Thus on the basis of above studies compound 2 was identified as 5-O-α-L Xylopyranosyl (1⟶6) β-D-glucopyranosyl 1,6 -dihydroxy 3,5 -dimethoxy xanthone (Figure- 2). It is the first reported compound in the family Celastraceae.

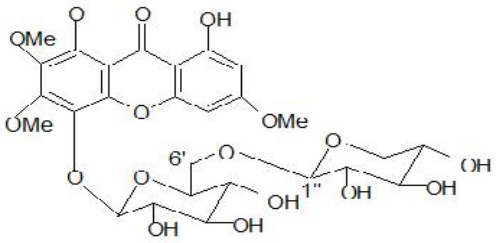

Figure -2

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Alterneriol: Secondary Metabolites Derived From Endophytic Fungi Alternaria spp Isolated From *Catharanthus roseus*

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Abstract- Endophytic fungi produce bioactive metabolites which play an essential role to provide protection to their host against attack by another pathogens and environmental factors. Isolation, identification and cultivation of endophytic fungi from medicinal plants are important for the production and characterization of bioactive fungal metabolites. Present study was aimed to isolate and characterize endophytic fungi from the medicinal plant, *Catharanthus roseus* collected from garden area of Muthi and Ramnagar forest in Jammu. Five samples of leaves, stem and bark were collected from *C. roseus*. The sample of leave, stem and bark were inoculated in three media PDA (Potato dextrose agar), YMA (Yeast maltose agar), WA (water Agar). A total of three strains of endophytic fungi (VRE–1, VRE-3, and VRE-4) were isolated using PDA media and YMA media. Out of these three strains, only one strain VRE-1 has been identified as *Alternaria spp*. by microscopically and biochemically. *Alternaria spp* further used for fermentation by using shake flask method and then the secondary metabolites were extracted by using Column Chromatography. One pure compound, Alternariol was obtained from methanolic extract, further study is undertaken.

Key words: Endophytic fungi, *Catharanthus roseus*, Secondary Metabolites, *Alternaria spp.*

Introduction

Fungi have been widely used as a source of bioactive compounds. The first milestone in this field was the discovery of penicillinnotatum by Alexander Fleming in 1928(Ligon B., 2004). Fungi are heterotrophic organisms and have different lifestyle; they live in mutualistic, antagonistic, or neutral symbiosis with a wide variety of autotrophic organism. Various effective antifungal agents like Griseofulvin, Cyclosporine, Mevastatin, Taxolare isolated from different fungus and used for the treatment of fungal infection (Stierle *et al*., 1993). The gold bioactive compound taxol discover from the endophytic fungus *Taxomycesandreanae* (Stierle *et al*., 1993). Endophytic fungi found in leaves, stem and bark of *Catharanthus roseus* having medicinal properties (Ansari and Naved., 2006). Fungus produce metabolites (primary and secondary) which play an important role in plant defense against herbivores and other interspecies defense. The primary and secondary metabolites produced by the fungi while some secondary metabolites are often derived from the primary metabolites and some environmental factors are responsible for the production of secondary metabolites which protect the fungi as well as plants from external factors (Vanisree lee *et al*., 2004). It is suggested that secondary metabolites also play an important role in chemical defense and communication of fungi. The present study was aimed to isolate the endophytic fungi from plants and also extracts the secondary metabolites produced by the entophytic fungi.

Material and Methods

Collection of samples: A total of five **s**amples were collected during January 2013. Leavesstem and bark of *Catharanthus roseus* were collected from Muthi area of Jammu (J&K) and Kangra Distt. (H.P.).

Isolation of endophytic fungi: Media used for the isolation of endophytic fungus were WA, YMA and PDA. Fungal growth was observed on the plates as white cottony outgrowth. The fungal growth was initiated within 7 to 10 days of inoculation. Isolation from the master plate was done by transfer of hyphal tips to PDA plates from water agar plates without addition of antibiotics to obtain pure culture.

Characterization of endophytes: The fungi were identified on the basis of their morphological and culture characteristics. Production of extracellular enzymes – amylase, protease, chitinase, lipase, oxidase and catalase was measured by biochemical analysis. Seed preparation on Potato Dextrose Agar (PDA) was done to obtain secondary metabolites. Bulk growth of endophytes on PDA was examined every day. The cell mass was separated out from the supernatant by the process of filtrations with the help of muslin cloth and kept the supernatant as well as biomass separately (Carroll, 1978).

Extraction of biomass: Extraction of endophytes from fermented broth was homogenized with tissue homogenizer and 10% methanol. The extraction procedure was done with organic solvent, chloroform. The compound which was left, bind to the methanol due to vigorous shaking. After 24 hours, separate the supernatant and biomass by filtration.

Drying of biomass by evaporation: The sample volume of the extracted solution to dryness containing metabolites of the fungal mycelium was done using rota- vapour. The main function of the rota- vapour is to separate the solvent from biomass.

Detection of secondary metabolites from *Alternaria spp.*: The secondary metabolites from endophytic fungi *Alternaria spp* was extracted by using thin layer chromatography. Then the separated compounds were purified by using Column chromatography. The physical and chemical properties of extracted compound was determined by nuclear magnetic resonance (NMR).

Results

Isolation: In this study three numbers of fungal endophytes (VRE-1, VRE-3, and VRE-4) were isolated from *Catharanthus roseus* as pure culture and these were isolated from stem and leaves of the plant. The VRE-1 isolate was identified as *Alternaria spp.* of endophytic fungi by Morphological (Table-1) and Biochemical analysis (Table-2). We have used WA, PDA, YMA media for the extraction of endophytic fungi. Out of these three media, PDA and YMA were found highly suitable for the isolation of endophytic fungi.

The present study revealed that the endophytic fungus isolated from *C. roseus* was characterized as *Alternaria spp*. These endophytes present in a plant produced several secondary metabolites which help in survival of endophytes as a commensal as well as these secondary metabolites have great medicinal value.

In the present study, secondary metabolite was extracted by column chromatography from methanolic extract of *Alternaria spp*. The melding point of the compound was 205.88 °C, physical state was solid and molecular weight was 258.23 g·mol^{-1}. The extracted bioactive compound was identified as Alternariol ($C_{14}H_{10}O_5$) (Figure) by NMR.

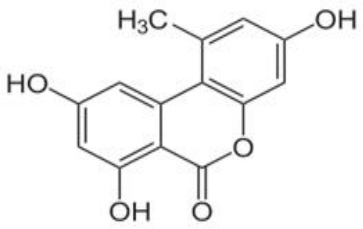

Figure- Chemical structure of Alternariol derived from Endophytic fungi *Alternaria spp.*

Discussion

Plant endophytic fungi are important and novel resource of natural bioactive compounds as they have wide potential applications in agriculture, medicine and food industries. In the past two decades, many

Table-1 Morphological and culture characteristics of endophytic fungal isolates isolated from *C. rosesus*(n=3)

S. No	Isolates	Colony Characteristics	Observation
1	VRE-1	Mycelia growth was observed and showed brown color, club shaped and highly separate	*Alternera spp.*
2	VRE-3	Mycelia growth with green colorization.	Unknown
3	VRE-4	Spores were observed and it was also green in color.	Unknown

Table-2 Biochemical analysis of endophytic fungal isolatesisolated from *C. rosesus*(n=3)(n=3)

Sample	protease	Amylase	Lypase	Chitinase	Oxidase	Catalase
VRE-1	Negative	Negative	Positive	Negative	Negative	Positive
VRE-3	Positive	Positive	Negative	Negative	Negative	Negative
VRE-4	negative	negative	Negative	Negative	Negative	Positive

valuable bioactive compounds have been extracted from the endophytic fungi as beneficial products having various antimicrobial, insecticidal, catatonic and anti cancer activities.

In the present study, a total five samples which include stem, leave and bark of *Catharanthus roseus* was collected from different areas. Out of five samples endophytic fungi were isolated from three samples of stem and leaves of *Catharanthus roseus*. The cultural characterization of fungal endophyte was performed using PDA, YMA and WA media. It was observed that the endophytes grow best on PDA medium in comparison to YMA and WA media. On the basis of these characteristics, three isolates VRE-1, VRE-2 and VRE-3 were obtained. The isolates were further screened for the presence of various enzymes i.e. amylase, chitinase, lipase, protease, oxydase and catalase using biochemical activity assay. After determining the morphological and biochemical observations, VRE-1 was identified as *Alternaria spp*., while VRE-3 and VRE-4 remains unidentified due to no similarity found with other fungal species. Earlier, a study conducted by Jagetia and Venkatesh (2005) reported the presence of 10 endophytic fungi from the family *Aegelmarmelose* while in the present study only one endophytic fungi of family *Alternatia spp.* were found. The fermentation of VRE-1 was performed by using shake flask method on PDB (Potato Dextrose Broth) after inoculating the pure culture and incubated at appropriate temperature. The extraction of the fermented broth was done with dichloromethane (DCM) to get both high polar and low polar compounds. The extract of *Alternaria spp*. was then subjected to column chromatography for isolation of pure compounds and one pure fraction was isolated. Then NMR of pure fraction was performed to know the structure of compound and the compound identified as alternariol. Alternariol was first isolated and structurally characterized as 3, 7, 9-trihydroxy-1-methyl-6H-dibenzo [b, d] pyran-6-one (Raistrick *et al*., 1953). Further research is required to explore the role and importance of Alternariol.

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Growth Hormones Stimulate the Enzyme Activities and Total Nitrogen Content in Salinity Stressed Seeds of *Triticum aestivum* cvs.

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Abstract-When salinity ($1x10^{-1}$M) stressed seeds of *Triticum aestivum* cv. PBW-502 and WH-711 treated with growth hormones (IAA, GA and Kn), an increased level of nitrogen and total activities of α- amylase, β-amylase, protease, acid phophatase and alkaline phosphatase were found. The content of protein and sugar was not altered.The response exhibited in order: IAA < Kn < GA < mixed (IAA + Kn + GA). However, these effects were cultivar and parameter specific.
Key words: α- amylase, β- amylase, protease, acid and alkaline phosphatase, IAA, Kn, GA.

Introduction

Soil, water salinity is known to cause considerable decifit in plant growth and yield. **(Ashraf 2009; Chaum *et al.*, 2011 ; Allakhverdiev *et al.*, 2000 ; Zhu, 2007).** The reduction in seed germination **(Das and Panda, 2001),** seedling growth **(Ashraf et al., 2002),** enzyme activities **(Seckin et al., 2009)** is also reported. Salts inhibit the physiological process by affecting the biochemical process such as N and CO_2 assimilation and protein biosynthesis **(Cusido et al., 1987).** Some report indicate that exogenous supply of IAA **(Khan et al., 2004),** GA **(Afjal et al., 2005)** and Kn **(Gul et al., 2000)** to plant can alleviate the adverse effect of salt and improve the germination, growth and yield (**Egamberdieva , 2009).** So, further to explore these experiments, the present studies has been carried out at biochemical level and enzyme activities in imbibed seeds of *Triticum aestivum* cv. WH-711 and PBW-502.

Material and Methods

The seeds of cereals – *Triticum aestivum cvs.* (PBW-502 & WH-711) with uniform size, shape, colour and weight as far as possible, were selected, surface sterilized with 0.1% $HgCl_2$ solution, thoroughly washed with distilled water and treated with inhibitory dose of NaCl and promotory dose of growth hormone i.e. IAA, GA and Kn and the combination of promotory dose of all these growth hormones for 24 hr. After imbibitions, the estimation of level of biochemical components (total sugar , reducing sugar, total protein and total nitrogen) and total activities of certain enzymes (α-amylase, β- amylase, protease, acid phosphatase and alkaline phosphatase) in these imbibed seeds was measured,

The estimation of total carbohydrate was done by Anthrone Method (**Hedge *et al.*, 1962).** The amount of carbohydrate was calculated in term of mg/gm dry weight with the help of calibration curve.

Estimation of reducing sugar: The estimation of reducing sugar was done by DNS method **(Bernfeld *et al.*, 1955).** The total reducing sugar was estimated in terms of glucose released in mg/gm fresh weight using glucose as a standard.

Estimation of Total Nitrogen: Estimation of nitrogen was done according to **Snell & Snell (1945).** The amount of nitrogen was calculated in terms of mg/gm dry weight.

Estimation of total protein: Protein estimation was done with the help of Lowry method **(Lowry *et al.*, 1951).** Protein was estimated in terms of mg/gm fresh weight.

Determination of enzyme activity: A common Tris – NaOH buffer at 6.8 pH was prepared. **(Vimla,1983).** This was used as extraction cum assay medium for amalyses and proteases. Crude enzyme was extracted by homogenising 1 gm material in 10 ml buffer and centrifuging the extract to get a clear supernatant, which was made to 20 ml with the buffer. The prepration constituted the crude enzyme extract. Further , each enzyme was assayed as per the method given here under**:**

~- amylase activity: Alpha amylase was assayed using 0.2 M tris-malate buffer of pH-6.8 was used as common extraction cum assay medium for amylase. Crude enzyme was extracted by homogenizing 50mg sample in 8 ml of buffer and centrifuging the extract at 5000rpm for 15 minutes to get a clear supernatant, which was made to 8ml with the buffer. Take 1ml of enzyme extract and 1ml substrate i.e. starch (0.15%) added to it and then incubate it at room temperature for 10 minutes. Now add 3ml of quinching reagent and read O.D. at 620nm with the help of spectrophotometer. Alpha- amylase activity was determined in term of mg starch degraded per minute per gram fresh weight **(Filner and Varner, 1967).**

- amylase activity: Pipette 0.5 ml of respective enzyme dilutions into a series of numbered test tubes. Incubated a blank with 0.5 ml distilled water. Incubated the tubes at 25°C for 3 to 4 minutes to achieved temperature equilibrium. After that added 0.5ml starch solution (1%). Incubated exactly 3 minutes and add 1ml DNS color reagent to each tube. Incubate

all tubes in a boiling water bath at 100°C for 5 minutes. Cool at room temperature and mix well then read absorbance at 540 nm using calibration curve of maltose (**Bernfeld, 1955).** Activity was determined in term of mg maltose degraded per minute per gram fresh weight.

Protease activity: 1 ml of enzyme extract was incubated for 1 hr at 40°C with 1 ml substrate (4mg/ml casein in buffer). The reaction was quenched by addition of 2 ml of TCA and chilling for 3 hr. The supernatant was collected by centrifugation, made slightly alkaline by addition of 1 ml 1.5 N NaOH and final volume made to 5ml with buffer. 1 ml of this was mixed with 5 ml of copper sulphate reagent after 10 minutes , 1 ml Folin's reagent (Lowry et al.) was added to the reaction mixture, kept for 30 minutes and then take O.D. at 620nm against blank. **(Yomo and Varner, 1973).** The amount of amino acids released , in terms of tyrosine was calculated. Activity was expressed as mg or µg tyrosine released / h / gm fresh weight.

Acid and alkaline phophatase: Crude enzyme was extracted by homogenizing 50mg plant material in extraction buffer and centrifuging the extract at 6000rpm for 15 minutes to get a supernatant. Now take 50 µl of sample and 25 µl of pNpp were added in it and then make up the volume by 2.925ml of acetate buffer (pH-5) for acid phophatase and tris buffer (pH-7.5) for alkaline phosphatase. Then incubate at 37°C for 30 minutes. After incubation, 2ml of 0.1N NaOH was added in it. After that O.D. was taken at 430nm with the help of spectrophotometer. The activity of acid phosphatase was determined by **(Lea, 1990; Prince *et al*., 1982; Wilson *et al*., 1996 and Sawhney 2007)** in terms of pNpp as a substrate at 430nm. Activity was determined in term of mg pNpp degraded/min / gram fresh weight.

Result and Discussion

Table **1** exhibit the interactive effect of $1x10^{-1}$M dose of NaCl and doses of growth hormones (i.e. $1x10^{-5}$M of IAA, $1x10^{-9}$M of Kn and $1x10^{-1}$M of GA) on level of biochemical component in imbibed seeds of *Triticum aestivum* cv. PBW-502. At the treatment of NaCl+IAA, the total protein content is 104%, total sugar is 102%, reducing sugar is 102% of control, total nitrogen is 146% of control, at the treatment of NaCl+GA, the total protein content is 105%, total sugar is 103%, reducing sugar is 103% of control, total nitrogen is 236% of control, at the treatment of NaCl+Kn, the total protein content is 105%, total sugar is 103%, reducing sugar is 103% of control, total nitrogen is 208% of control and at the treatment of NaCl+IAA+GA+Kn, the total protein content is 109%, total sugar is 106%, reducing sugar is 107% of control, total nitrogen is 364% of control. Increase is maximum in total nitrogen content at NaCl + all growth hormone, a slight stimulation in other parameters.

Table-1 Interactive effect of NaCl dose ($1x10^{-1}$M) and growth hormone doses ($1x10^{-5}$M IAA,$1x10^{-1}$M GA and $1x10^{-9}$ M Kn) on the level of biochemical component in imbibed seed of *Triticum aestivum* cv.PBW-502

Treatments → / ↓ Parameters		control	NaCl	NaCl+ IAA	NaCl + GA	NaCl + Kn	NaCl+ IAA+GA+ Kn
Level of biochemical components	Protein Content (mg/g fresh weight ±SD)	82.14±1.34	80.16±8.42	83.12±8.64	84.22±7.69	84.44±7.48	87.24±9.47
	Total Sugar (mg/g Dry Weight ± SD)	138.11±17.69	137.5±13.09	140.22±14.23	141.54±14.29	141.98±14.49	145.87±15.2
	Reducing Sugar (mg/g fresh weight ± SD)	89.37±9.84	88.79±8.78	90.44±9.72	91.12±9.87	91.47±9.71	95.32±9.82
	Total Nitrogen (mg/g Dry weight ± SD)	0.66±0.08	0.78±6.12	1.14±0.36	1.84±0.49	1.62±0.43	2.84±0.89

Table- **2** indicate the interactive effect of $1x10^{-1}$M dose of NaCl and doses of growth hormones (i.e. $1x10^{-6}$M of IAA, $1x10^{-8}$M of Kn and $1x10^{-7}$M of GA) on level of biochemical component in imbibed seeds of *Triticum aestivum* cv. WH-711. On the treatment of NaCl+IAA, the total protein content is found 102%, total sugar 101%, reducing sugar 102% of control, total nitrogen 105% of control, at the treatment of NaCl+GA, the total protein content 104%, total sugar 102%, reducing sugar 103% of control, total nitrogen 112% of control, at the treatment of NaCl+Kn, the total protein content 104%, total sugar 102%, reducing sugar 104% of control, total nitrogen 109% of control and the total protein content 107%, total sugar 106%, reducing sugar is 109% of control, total nitrogen is 144% of control on the at the treatment of NaCl+IAA+GA+Kn.

It indicates that *T. aestivum* cv. PBW-502 is more sensitive than cv. WH-711 in response to interaction of salinity and growth hormones. These are comparable to other studies also. **Kim *et al* (2006)** have reported that the total sugar and reducing sugar are increased when NaCl stressed seeds of different plants are treated with IAA and GA in rice. Similar study is reported by **Sadak *et al* (2013)**, when salt stressed faba bean seed treated with IAA and Kn treatment gives positive effect and total carbohydrate, free amino acid, proline and phenolic compounds are increased .

Table-2 Interactive effect of NaCl dose ($1x10^{-1}$M) and growth hormone doses ($1x10^{-6}$M IAA,$1x10^{-7}$M GA and $1x10^{-8}$ M Kn) on the level of biochemical component in imbibed seed of *Triticum aestivum* cv.WH-711

Treatments → / ↓ Parameters		Control	NaCl	NaCl+ IAA	NaCl + GA	NaCl + Kn	NaCl+ IAA+GA+ Kn
Level of biochemical components	Protein Content (mg/g fresh weight ±SD)	140.16±0.88	138.11±13.14	141.24±14.23	143.12±14.17	143.24±14.03	147.54±14.32
	Total Sugar (mg/g Dry Weight ± SD)	150.88±12.3	148.47±15.22	150.47±15.27	152.14±15.20	151.78±15.36	156.87±16.11
	Reducing Sugar (mg/g fresh weight ± SD)	75.78±9.73	74.65±7.42	76.41±6.76	77.14±7.31	77.84±7.79	81.27±8.33
	Total Nitrogen (mg/g Dry weight ± SD)	2.32±0.95	2.86±0.23	2.99±0.13	3.21±0.34	3.11±0.42	4.11±0.70

Table- **3** shows the interactive effect of $1x10^{-1}$M dose of NaCl and doses of growth hormones (i.e. $1x10^{-5}$M of IAA, $1x10^{-9}$M of Kn and $1x10^{-1}$M of GA) on activities of certain enzyme in imbibed seeds of *Triticum aestivum* cv. PBW-502. On the treatment of NaCl+IAA, the total activity of α-amylase is 125%, β-amylase activity 120%, protease activity 133%, acid phosphatase activity 130% and alkaline phosphatase activity 122% of control, on the treatment NaCl+GA, , the total activity of α-amylase is 141%, β-amylase activity 130%, protease activity 147%, acid phosphatase activity 155% and alkaline phosphatase activity 113% of control, on the treatment of NaCl+Kn, , the total activity of α-amylase 144%, β-amylase activity 134%, protease activity 156%, acid phosphatase activity 182% and alkaline phosphatase activity 107% of control and while on the treatment of NaCl+IAA+GA+Kn, the total activity of α-amylase 176%, β-amylase activity 157%, protease activity 208%, acid phosphatase activity 234% and alkaline phosphatase activity 178% of control respectively

Table-3 Interactive effect of NaCl dose ($1x10^{-1}$M) and growth hormone doses ($1x10^{-5}$M IAA, $1x10^{-1}$M GA and $1x10^{-9}$ M Kn) on total activity of certain enzymes in imbibed seed of *Triticum aestivum* cv.PBW-502

Treatments →		control	NaCl	NaCl+ IAA	NaCl + GA	NaCl + Kn	NaCl+ IAA+GA+ Kn
Activities of Enzymes (Per gm fresh weight ± SD)	α-amylase activity (mg starch degraded/min.)	8.84±6.09	8.19±0.86	10.24±0.18	11.54±0.11	11.78±0.32	14.41±0.16
	β- amylase activity (mg maltose degraded/ min.)	7.44±6.05	7.36±0.75	8.84±0.45	9.54±0.85	9.88±0.61	11.59±0.16
	Protease activity (mg tyrosine released/hr.)	7.84±0.44	6.29±0.53	8.41±0.81	9.22±0.79	9.84±0.28	13.11±0.18
	Acid phosphatase activity (mg pNPP degraded/min.)	6.54±3.56	4.79±0.22	6.22±0.54	7.41±0.48	8.74±0.64	11.21±0.17
	Alkaline phosphatase activity (mg pNPP degraded/min.)	7.89±0.22	7.56±0.54	9.21±0.90	8.54±0.88	8.1±0.46	13.51±0.14

Table- **4** indicate the interactive effect of $1x10^{-1}$M dose of NaCl and doses of growth hormones (i.e. $1x10^{-6}$M of IAA, $1x10^{-8}$M of Kn and $1x10^{-7}$M of GA) on activities of certain enzyme in imbibed seeds of *Triticum aestivum* cv. WH-711. On the treatment of NaCl+IAA, the total activity of α-amylase is 104%, β-amylase activity 105%, protease activity 138%, acid phosphatase activity 142% and alkaline phosphatase activity 123% of control. On the treatment NaCl+GA, , the total activity of α-amylase 102%, β-amylase activity 101%, protease activity 272%, acid phosphatase activity 180% and alkaline phosphatase activity 149% of control. On the treatment of NaCl+Kn, , the total activity of α-amylase 104%, β-amylase activity 103%, protease activity 354%, acid phosphatase activity 192% and alkaline phosphatase activity 137% of control and on the treatment of NaCl+IAA+GA+Kn, , the total activity of α-amylase 111%, β-amylase activity 112%, protease activity 474%, acid phosphatase activity is 217% and alkaline phosphatase activity is 183% of control respectively.

Similarly, **Kim *et al.* (2006)** have also reported that the activity of α-amylase increased when NaCl stressed seeds of different plants is treated with IAA and GA in rice. The effects of plant hormones on seed germination, researchers have found that both under stress and non-stress conditions, N compounds, including nitrous oxide can enhance seed germination through enhancing amylase activities **(Zhang *et al.*, 2005; Hu *et al.*, 2007; Zheng *et al.*, 2009)**. Through decreasing the production of O_2 and H_2O_2 such products can also alleviate the stress by controlling

the likely oxidative damage, similar to the effects of antioxidant enzymes including superoxide dismutase (SOD), catalse (CAT) and peroxidase (POD) on plant growth undervarious stresses **(Song *et al.*, 2006; Tian and Lei, 2006; Tseng *et al.*, 2007; Li *et al.*, 2008; Tuna *et al.*, 2008; Zheng *et al.*, 2009; Sajedi *et al.*, 2011).** Increase of enzyme activities still remains a question, it needs further kinetic studies.

Table-4 Interactive effect of NaCl dose ($1x10^{-1}$M) and growth hormone doses ($1x10^{-6}$M IAA, $1x10^{-7}$M GA and $1x10^{-8}$ M Kn) on total activity of certain enzymes in imbibed seed of *Triticum aestivum* cv.WH-711

Treatments →		control	NaCl	NaCl+ IAA	NaCl + GA	NaCl + Kn	NaCl+ IAA+GA+ Kn
Activities of Enzymes (Per gm fresh weight ± SD)	α-amylase activity (mg starch degraded/min.)	31.51±6.53	30.8±3.18	32.14±3.38	31.48±3.26	31.89±3.33	34.33±3.28
	β- amylase activity (mg maltose degraded/ min.)	25.21±5.76	25.96±2.25	27.41±2.22	26.12±2.16	26.89±2.25	29.15±2.24
	Protease activity (mg tyrosine released/hr.)	1.69±0.85	0.81±0.75	1.12±0.14	2.21±0.24	2.87±0.41	3.84±0.36
	Acid phosphatase activity (mg pNPP degraded/min.)	1.13±0.74	1.56±0.14	2.21±0.24	2.81±0.23	2.99±0.20	3.38±0.36
	Alkaline phosphatase activity (mg pNPP degraded/min.)	4.31±0.69	4.29±0.40	5.28±0.36	6.41±0.55	5.88±0.65	7.84±0.70

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Phytochemical Screening and Antimicrobial Activity of *Zanthoxylum armatum DC.*

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Abstract- *Zanthoxylum armatum* is used in traditional medicinal systems for number of disorders in Uttarakhand and other parts of India. The antimicrobial activity of different extracts of plant-stem bark in different solvents (water, ethanol, methanol, hexane) were tested against 8 pathogenic bacteria and 3 isolated fungal strain by disc diffusion method. Two extracts of bark show mild antibacterial activity and limited antifungal activity but methanolic and aqueous extract gives wide range antibacterial activity against some pathogenic bacteria. MICs of methanol extract of *Z. armatum* root produced best inhibition zones against *P. Mirabilis* (18mm), *S. Mutons*(20mm), *Gardoni* (18mm) and aqueous extract produce inhibition zone against *Listeria*(18mm) and Gardoni (20mm). Chemical analysis revealed the presence of terpenoid, saponin and reducing sugars as major compounds in the stem bark of the plant.

Key words: *Zanthoxylum armatum,* antibacterial, antifungal activities, inhibition zone, stem bark.

Introduction

India has richest plant based medicinal traditional system because of its rich biodiversity. These herbal medicines are mainly used for health care due to their cost value, effectiveness and lesser side effects on human body. Among different medicinal plants of The Indian Himalayan region (IHR), *Zanthoxylum* (family: Rutaceae) is one of such genus which possess high medicinal importance and have about 250 species spreading all over the world. In India, 11 species of this genus is reported and 4 species; Z. *armatum* DC (Syn. Z. *alatum* Roxb.), *Z. acanthopodium* DC. Z. *oxyphyllum* Edgew, and Z. *budrunga* are present in Uttarakhand. *Zanthoxylum armatum* is widely distributed in India from Kashmir to Bhutan upto 2500m altitudes. The bark is utilized as traditional dye yielding resource. Chemical studies carried out on *Zanthoxylum* species have revealed the occurrence mainly of alkaloids, flavonoids, terpenoids, aliphatic and aromatic amides, lignans, coumarins, sterols, carbohydrate residues etc. Some of these metabolites have reported, antibacterial, antifungal, antioxidant, cytotoxic, molluscicidal, anti-sickling, anesthetic anti-hypertensive and anti-inflammatory properties.

Material and Methods

Preparation of extract: The extracts of medicinal plants were prepared by dissolving sample in 1:10 with different solvents separately (Distilled water, ethanol, methanol and hexane respectively) and a constant heating was provided by heating mental for 6 hours in soxhlet appratus. Then extracts in round bottom flask were transferred to pre-weighted apenldrof. Apenldrof containing extracts were weighted and noted down and finally, the percentage yield was calculated. Then the extracts were stored at 4°C.

Yield of extract= Weight of empty apendrof – weight of apendrof with sample

$$\%\text{yield} = \frac{\text{Yield of extract}}{\text{Weight of raw material taken}} \text{ X } 100$$

Bacterial Culture: The human pathogenic bacteria such as *Staphylococcus* sp., *Escherichia coli, Listeria, P. Vulgaris, S.Gardoni, P. Mirabilis, S.Mutons, Clostridium* were obtained from Bioinformatics Centre, IMTECH, Chandigarh and were maintained in Nutrient agar slant at 4°C for experimental studies. The fungus strain of *Penicillin* was isolated from Potato Dextrose Agar.
Preparation of standard culture inoculum of test organism: The colonies of different bacteria and strain of Fungus were inoculated in the 20 ml nutrient broth and incubated for 24 hours and 72 hours respectively.
Assay of antimicrobial activity: Assay of Anti bacterial activity done by Disc Diffusion method (Kirby-Bauer method, 1966). All plates were incubated at 37°C for 24 h. Zone of inhibition was noted down. Each experiment was performed 3 times. Discs

impregnated with only solvents were used as negative controls.

Antifungal assays: Assay of Anti Fungal activity was done by using disc diffusion method (Kirby-Bauer method, 1966). Each experiment was performed 3 times.

Minimum Inhibitory Concentration (MIC) Assay: The MIC method was applied on extracts that proved their high efficacy against microorganisms by serial dilution method. All plates were incubated at 37°C for 24 h. Zone of inhibition was noted down. Each experiment was performed 3 times.

Phytochemical studies: Chemical Tests were carried out on the ethanol, methanol, water and –hexane extracts of *Zanthoxylum Armatum* plant using standard procedures (Abdul Wadoodet al.,2013 and Pravin S. Jogi et al.,2012).

Results and Discussion

The percentage yields of extracts and the phytochemical constituents of the plants are shown in Table 1. Among all the extracts the Aqueous extract of bark of *Zanthoxylum Armatum* contain the higher amount of soluble solids followed by ethanol, methanol and hexane.

Table- 1 The yields of plants extracted in different solvents.

Plant part	Extraction yield (%)			
	Water	Ethanol	Methanol	Hexane
Bark	26.8	11.62	3.82	1.39

Antibacterial activity: The methanolic extract of plant only shows some antibacterial properties against *Mirabilis* (18mm), *Mutons* (20mm) and *Gardoni*(18mm) while aqueous extract shows zone of inhibition for *Listeria(18mm)* and *Gardoni*(20mm) shown in table 2. Barkatullah et al. in 2012 reported that the ZLE extract showed highest inhibition against *M. leutus* (18.00 ± 0.71 mm), *P. multocida* (18.00 ± 0.71 mm), *E. coli* (17.00 ± 0.71) and *B. subtilis* (15.33 ± 0.81 mm). The ZFE showed topmost action against *M. leutus* (21.33 ± 0.41 mm) and *P. multocida* (18.33 ± 0.41 mm) while the ZFH showed inhibitory action against *M. leutus* (19.67 ± 0.41 mm) as compared to other tests species. The ZBH extract was found active against *M. leutus* (20.33 ± 0.41 mm). The minimum inhibitory concentration (MIC) values for most of the bacterial species were found to be 0.65 µg/ml. The crude ethanolic and *n*-hexane of all parts were proved a rich source of fungicidal effect. Highest flavonoids was found in ethanolic extract of *Z. armatum* fruit (ZFE) (22.8 ± 1.33 mg/g) followed by ethanolic extract of *Z. armatum* bark (ZBE) (18.33 ± 1.22 mg/g) while highest phenolic contents were found in ZFE (21.68 ± 0.44 mg/g) followed by ZBE (16.48 ± 1.33 mg/g).

Table- 2 Zone of Inhibition (in mm) of bark extracts of *Zanthoxylum Armatum* against Gram-positive and Gram-negative bacteria at different concentration.

Bacteria tested	Ethanol extract (mg/ml)			Aqueous extract (mg/ml)			Methanol extract (mg/ml)			Hexane extract (mg/ml)		
	100	50	10	100	50	10	100	50	10	100	50	10
E.coli	7	8	8	8	6	6	10	7	6	6	7	7
Clostridium	8	8	6	6	7	8	8	8	7	7	6	7
Listeria	7	8	7	18	9	9	7	7	6	9	7	8
S. Gardoni	7	7	6	20	13	10	18	12	10	9	8	8
S. Mutons	8	8	7	7	8	7	20	17	15	6	8	7
P.Mirabilis	8	7	8	7	7	6	18	16	11	6	7	8
P.Vulgaris	8	8	7	7	6	8	6	8	8	7	6	8
Staphylococcus	7	7	7	8	7	7	7	7	8	8	7	7

Antifungal activity: The fungus strain *Arthrogrophis Cuboida* infect eyes, central nervous system and cause nail infection i.e. onychomycosis; *A. fumigatus* causes immunodeficiency and *Aspergillus niger* which causes Aspergillosis, a serious lung infection, if large amounts of spores are inhaled. The antifungal activity of Z. armatum bark extracts give very low inhibition zone against isolated fungal strain except hexane extract. Small inhibition Zone was in methanolic extract was found against *A. Fumigatus* (17mm) shown in table 3. Recently, Rakesh Raturi et al. (2014) studied the antifungal activity of stem bark of *Z. armatum* plant against another fungal bacterial *Fusarium oxysporum.*

Table- 3 Zone of inhibition (mm) for antifungal activity of *Z. armatum* plant

Fungus strain	Stem bark			
	Aqueous extract	Ethanolic extract	Methanolic extract	Hexane extract
Arthrogrophis Cuboida	8	10	12	7
Aspergillus Fumigatus	7	13	17	6
Aspergillus Niger	9	11	14	6

Phytochemical analysis: In present study, we have performed for different phytochemicals like alkaloids, tannins, flavanoids, terpinoids, reducing sugar, saponin, phlobatannins in different extracts (water, ethanol, methanol and hexane) which is shown in table 4. The methanolic extract of bark of plant shows maximum amount of constituents present compare to others. In methanolic extract, terpenoid and reducing sugar are present in maximum quantity followed by Flavanoid then alkaloid and saponin. Hexane extract contain no phytochemical constituents

Table- 4 Preliminary phytochemical analysis of screened bark of *Zanthoxylum Armatum*

Phytochemicals	Water	Methanol	Ethanol	Hexane
Flavonoid	-	++	+	-
Terpenoid	-	+++	-	-
Alkaloid	+	+	++	-
Reducing sugar	+++	+++	-	-
phlobatannins	-	-	+	-
Tannine	-	-	+	-
Saponin	+	+	+	-

+ = indicates presence of phytochemicals and

- = indicates absence of phytochemicals.

+++ = shows high concentration.

++ = shows moderate concentration.

Conclusion

The present study deals with the antibacterial, antifungal activity and phytochemical screening of *Z. armatum.* This plant has many medicinal uses especially as antibacterial agent due to the presence of medicinal compounds. On the basis of results of present study we have concluded that the methanolic extract of stem bark of plant possess antibacterial activity against *S. Gardoni* (18mm), *S. Mutons (20mm)* and *P.Mirabilies (18 mm)*. The stem bark of plant shows unsatisfactory result against *Arthrogrophis Cuboida, A. Niger and A. Fumigantus.* So, *Z. armatum* plant have limited antibacterial and antifungal activity against pathogenic bacteria. From phytochemical screening it concluded that the plant bark contain those compounds which are responsible for medicial values like reducing sugar, terpenoid, saponin as a major compound and other constituents like alkaloid and flavonoid.

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Comparison of Physicochemical Properties of Some Edible Oils

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Abstract- Oils and fats are important parts of human diet. These are the rich sources of dietary energy and contain more than twice the caloric value of equivalent amount of sugar. Their functional and textural characteristics contribute to the flavour and palatability of the natural and the prepared foods. They contain certain fatty acids which play an important role in nutrition and are also carrier of fat soluble vitamins. In the present paper, we have experimentally determined the saponification value, iodine value, acid value, and peroxide value for three different edible oils. The iodine value (IV) indicates the degree of unsaturation of the oil. The acid value (AV) give an idea of the amount of free acid present in the oil. The peroxides value (PV) gives the initial evidence of rancidity in unsaturated fats and oils. It gives a measure of the extent to which oil has undergone primary oxidation. The Saponification value (SV) is expressed by the amount of potassium hydroxide in mg required to saponify one gram of fat. It was observed on the basis of said values that soyabean oil and mustard oil showed higher values of unsaturation and auto-oxidation than those given by olive, coconut and pea-nut oils.

Key words: Edible oils, Iodine value, Acid value, Saponification value, Peroxide value.

Introduction

Edible oils are triglyceride extracted from a plant. Such oils have been part of human culture for millennia. The term edible oil can be narrowly defined as referring only to plant oils that are liquid at room temperature, or broadly defined without regard to a substance's state of matter at given temperature (D.Robin,1999). The estimation of the physico-chemical properties of edible oils is essential in the design of unit processes such as distillation, heat exchangers, reactors and piping. On the other side, physico- chemical properties are an important factor that determines the overall quality and stability of a food system.). Density, saponification value, iodine value, acid value, peroxide values are some of the important characteristics of a vegetable oil. The coconut (*Cocosnucifera L.)* is an important fruit tree in the tropical regions and the fruit can be made into a variety of foods and beverages. Solid at room temperature, coconut oil is white and buttery in texture; it has a distinctive coconut flavor and is widely used in South India. Soya bean oil (*Glycine max*) is a vegetable oil extracted from the seeds of the soya bean. It is one of the most widely consumed cooking oils and is an important source of dietary protein widely used in a variety of dishes by oriental people for many centuries. The sunflower, (*Helianthus annuus L.*) seed itself is edible and its oil is used throughout the world for frying and cooking. It is also used as poultry feed. Peanuts (*Arachishypogaea L.)*are a very good source of monounsaturated fats, the type of fat that is emphasized in the heart-healthy Mediterranean diet. The olive tree (*Oleaeuropea L.)* is one of the most cultivated fruit trees since ancient times (Fabbri, 2006).Olive oil is a natural green vegetable oil which is abundant in vitamin, carotene and many trace elements and has many functions in nutrition and health care (Mar´ýa-Isabel Covas, 2006). Mustard Oil (*Brassica nigra*) is extracted at a low pressure and low temperature (40-60°C). It contains 0.30-0.35 % essential oil *(AllylIso- Thiocynate*) which acts as preservative. Ghee, the most famous traditional dairy product in India, many countries and the Middle East, is made from milk, cream, or butter of several animal species (G. S. Rajorhia, 2003).

Therefore, the aim of the present investigation was to ascertain the saponification, iodine, acid and peroxide value of these oils as a means to compare the physicochemical properties of the oil mentioned.

Material and Methods

Collection of oils: Edible oil viz., olive oil, peanut oil, mustard oil, soya bean oil, sunflower oil, coconut oil and desi ghee were collected from local areas of Dehradun.

Saponification Value: The saponification value of oil is defined as the number of milligram of KOH required to neutralize the fatty acid resulting from the complete hydrolysis of 1gm of oil or fat .The magnitude of its value gives an idea about the average molecular weight of the oil or fat. The higher the molecular weight of a fat, the smaller is its saponification value .The higher the saponification value, the greater is the percentage of the short chain acids present in the glycerides of the oil and fat.

Iodine value: The iodine value of an oil or fat is defined as the no. of grams of iodine taken up by 100 gm of the oil. Iodine value is an indication of the degree of unsaturated monochloride in acetic acid with the oil

dissolved in $CHCl_3$ or carbon tetra chloride and then back titrating the residual iodine with a standard sodium thiosulphate solution taking starch as the indicator.

Acid value: The acid value of oil is defined as the no. of mg of potassium hydroxide required to neutralize 1g of oil or the no of mg of KOH neutralized by the free acid presents in 1gm of oil. Such a value gives an idea of the amount of the free acids presents in the oil under study. Normally fresh and recently extracted oil or fats have very little or no free fatty acid present.

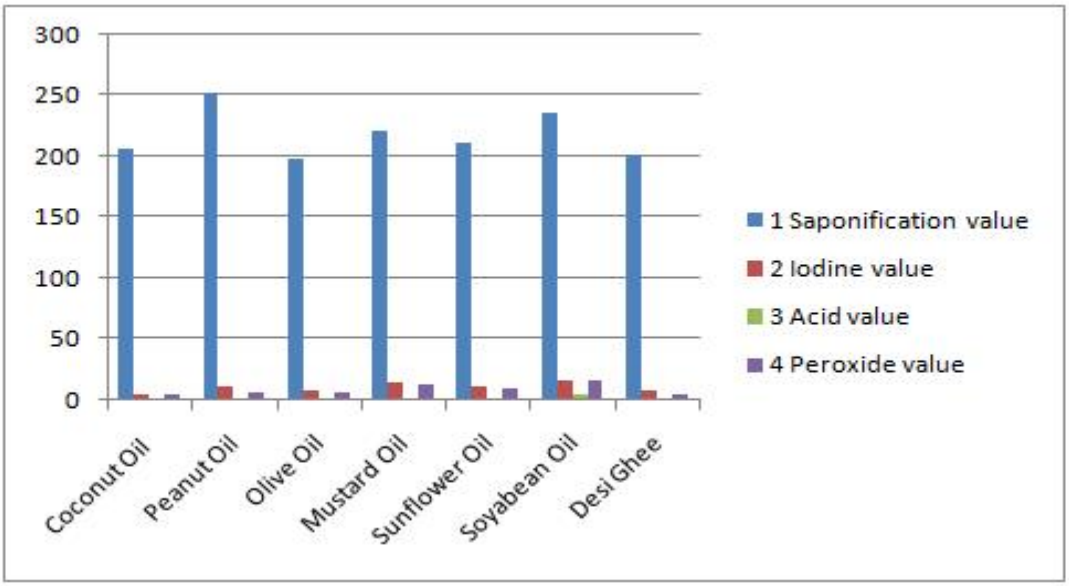
Peroxide value: Peroxide value (PV) measures the milli equivalents of oxygen (hydro peroxides) per 1000 gram of oil. The peroxide value is a measure of the concentration of substances that oxidize potassium iodide to iodine. The amount of free iodine is determined by titration with sodium thiosulphate using starch as indicator. A corresponding blank reagent is simultaneously prepared.(P.C.kamboj,2007)

Results and Discussion

The aim of this work was to characterize different edible oils on the basis of saponification, iodine, acid and peroxide values. The values obtained are given in the following table.

Table

S.No.	Properties	Coconut Oil	Peanut Oil	Olive Oil	Mustard Oil	Sunflower Oil	Soyabean Oil	Desi Ghee
1	Saponification value	206.4	252	198	221.6	211.6	235.6	201.6
2	Iodine value	5.7	11.8	9	14.6	11.3	15.6	7.6
3	Acid value	0.39	0.28	0.33	1.2	1.4	5.6	0.33
4	Peroxide value	4.66	5.8	5.9	12.6	10.3	15.6	5.6



Graph Physico-chemical properties of respective oils tested

Saponification value is a measure of oxidation during storage. It also indicates deterioration of the oils. From the above table, it can be seen that peanut oil has the highest value of SV while olive oil has the lowest among the oils tested. These results are in agreement with the studies conducted by Naegu, *et al.,* 2013.

The higher the iodine value, the less stable is the oil and is more vulnerable to oxidation and free radical production. High iodine value oils are prone to oxidation and polymerization. During heating, such as when used in cooking, oils with high iodine value readily oxidize and polymerize. Polymerization is an irreversible process which causes the fatty acids to become hard, insoluble, plastic-like solids. We have observed that highest iodine value is of soya bean oil followed by mustard and peanut oil.

Acid Value is a measure of the content of free fatty acids in the vegetable oil and describes the quantity of caustic potash solution which is necessary for the neutralization of the free fatty acids. The acid number depends strongly on the refining degree and the aging degree of oil. Since the acid value of soyabean oil is high, it depicts less comparative refining of soyabean oil as compared to other oils tested.

Detection of peroxides gives the initial evidence of rancidity in unsaturated fats and oils. It gives a measure of the extent to which oil has undergone primary oxidation. The double bonds found in fats and oils play a role in auto-oxidation. Oils with a high degree of unsaturation are most susceptible to auto-oxidation. Peroxides are intermediates in the auto-oxidation reaction. Here, we found that peroxide value of soya bean is highest followed by mustard oil and sunflower oil indicative of it being undergone auto-oxidation.

From the above values it can be concluded that soyabean oil followed by mustard oil and sunflower oil exhibit a higher level of unsaturation and auto-oxidation as compared to that of olive, cococnut and peanut oil. These results are in agreement with the studies conducted by Tasic et al., 1999 who observed lowest value of saponification for olive oil.

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Qualitative Phytochemical Analysis of *Allium humile* and *Ocimum gratissimum* (Linn.) Leaves Extract

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Abstract- The present study has been designed to analyse the phyto-constituents of various extracts of *Allium humile* and *Ocimum gratissimum* (Linn.) leaves. Various extracts of *A. humile* and *O. gratissimum* (Linn.) leaves were prepared in viz. Pet.ether, chloroform, acetone and methanol (12 hrs each) using Soxhlet extraction technique to get four extracts. These extracts were then subjected to qualitative chemical tests for the identification of various phyto-constituents viz, alkaloids, carbohydrates, glycosides, proteins, tannins, sterols, saponins, amino acids etc. This study demonstrates that methanolic extract of *Allium humile* leaves was rich in alkaloids, saponins, carbohydrate, glycosides, proteins and amino acids, whereas the chloroform extract of *O. gratissimum* (Linn.) gave test for alkaloids, triterpenoids, carbohydrates and glycosides. These results suggest that these phyto-constituents may be responsible for pharmacological activities of these plants.

Key words: *Allium humile*, *Ocimum gratissimum* (Linn.), Phyto-constituents, Extraction.

Introduction

Over the past few decades, the traditional knowledge on the use of medicinal plants has been widely acknowledged across the world. According to the World Health Organization, 80%of the world's population in developing countries uses traditional medicine[1]. In India, the knowledge of traditional herbal medicine is synonymous with its rich cultural heritage and has found its mention in Vedic literature, particularly Rigveda, Charak Samhita and Susruta Samhita. In the North of India, the Himalayas covering 18% of the Indian subcontinent accounts for more than 50% of India's forest and contains 30% of India's economic species. The area harbours about 8,000 species of higher plants of which 1,748 are used for medicinal purposes[2].Roughly 40% of plants provide active ingredients for modern drugs, and because of their use in traditional medicines, they draw the interest of researchers. Furthermore, the traditional knowledge with its holistic and systematic approach supported by experimental base can serve as an innovative and powerful discovery engine for newer, safer and affordable medicines.

Small Alpine Onion *Allium humile (A.nivale; A.gowanianum)*, belongs to family Alliaceae, found at high altitudes in the Himalayas at altitudes of 3000-4000 m. It is found on open alpine slopes from Pakistan to W. Nepal and China[3]. *Allium humile* is used as a condiment with pulses, blood purification, swelling, asthma, stomach disease, cold, cough, jaundice. The leaf and bulb parts are used locally in alleviation of inflammation and pain full conditions[4]. Leaves and inflorescences are used as seasoning agents. Few research articles reported the antibacterial activity[5]. *Ocimum gratissimum* (Linn.) is well-known plant used in the Indian system of medicine. A lot of research has been carried out on *O. gratissimum* (Linn.). Which is herbaceous plant (Family- Labiatae), indigenous to tropical areas especially India and in West Africa. In Nigeria, it is found in the Savannah and coastal areas. It is cultivated in Ceylon, South Sea Islands, and also within Nepal, Bengal, Chittagong and Deccan[6]. The plant is commonly used in folk medicine to treat different diseases such as upper respiratory tract infections, diarrhoea, headache, diseases of the eye, skin diseases, pneumonia, cough, fever and conjunctivitis[7]. In the present study, the various solvent extracts of *A.humile* and *O.gratissimum* (Linn.) were subjected to qualitative chemical tests for the identification of various phyto-constituents which are solely responsible for biological activity of these plants.

Material and Methods

Plant material: Leaves of *Allium humile* were collected from Chamoli. The plant material was identified from Botanical Survey of India, Northern Regional Centre, Dehradun, India with the reference number BSI/NRC 9 (Tech.)/2010-03/839/12796. Leaves of *Ocimum gratissimum* (Linn.) was collected from Indian Institute of Integrative Medicine, Jammu (Formerly

Regional Research Laboratory), CSIR, India. The reference number was [RRL(J)OG-14]. The leaves of *Allium humile* and *Ocimum gratissimum* (Linn.) were dried in shade at room temperature and ground separately at controlled temperature.

Chemicals and reagents were of analytical grade- The solvents and chemicals were of analytical grade. Petroleum ether, Chloroform, Acetone, Methanol, Ethyl acetate were purchased from Merck India Ltd., Mumbai. Benedicts reagent, Fehling reagent, Mayer's reagent, Dragendroff reagent, Hager reagent, Wagner reagent, Molish reagent, Millon reagent, Ninhydrin reagent, NaOH pellets, $FeCl_3$, were purchased from Ranbaxy Fine Chemicals Ltd, New Delhi. Picric acid and Hydrochloric acid was purchased from S.D. Fine Chemicals Ltd. Mumbai. α-naphthol, H_2SO_4 was purchased from Merck India Ltd. Mumbai.

Preparation of extracts: The fresh leaves of *A. humile* and *O.gratissimum* were dried in shade and at room temperature for 2 days followed by tray drying [40-50°C] for 3-4hrs and powdered to obtain coarse powder. 980g of powder of *A. humile* leaves and 2 kg *O. gratissimum* leaves were extracted with pet ether, chloroform, acetone and methanol (12 hrs each) using Soxhlet extraction technique to get four extracts. The solvent was removed by evaporation under reduced pressure to obtain a semisolid mass. The resultant concentrated extracts were then scooped into a pre-weighed small sterile container and weighed. The difference between the two weights was recorded as the percentage yield. The containers were then covered, labelled accordingly and the resultant extract was kept in desiccators[8].

Qualitative phytochemical analysis: The various solvent extracts of *A.humile* and *O.gratissimum* (Linn.) were subjected to qualitative chemical tests for the identification of various phyto-constituents viz, alkaloids, carbohydrates, glycosides, proteins, tannins, sterols, saponins, amino acids etc. These phyto-constituents are solely responsible for biological activity of these plants[9-11].

Results and Discussion

Qualitative phytochemical constituents of *Allium humile* leaves: All the extracts were screened for phytochemical constituents. Results showed the presence of triterpenoids in pet. ether extract; alkaloids, carbohydrates and glycosides were found in acetone extract, while methanol extract was rich in alkaloids, saponins, carbohydrate, glycosides, proteins and amino acids (Table-1).

Table-1 Results of Phyto-constituents present in different extracts of *Allium humile* leaves

Test performed	Pet. Ether Extract	Chloroform Extract	Acetone Extract	Methanol Extract
Test for Alkaloids				
Mayer's test	-	-	-	++
Dragendroff's test	-	-	-	++
Hager's test	-	-	++	++
Wagner's test	-	-	-	++
Test for Carbohydrates and glycosides				
Fehling test	-	-	++	++
Molish test	-	-	++	++
Benedict test	-	-	++	++
Test for phenolic compounds and Tannins				
Dil. $FeCl_3$-test	-	-	-	-
Test for sterols / Triterpenoids				
Salkowaski test	++	-	-	-

Liberman- Burchard's test	++	-	-	-
Test for Saponins				
Saponins Test					
Test for Proteins and Amino acids				
Ninhydrin test	-	-	-	++
Biuret test	-	-	-	++

(-) Absence, (++) Presence

Table- 2 Results of Phyto-constituents present in different extracts of *Ocimum gratissimum* (linn.) leaves

Test performed	Pet. Ether Extract	Chloroform Extract	Acetone Extract	Methanol Extract
Test for Alkaloids				
Mayer's test	-	-	++	++
Dragendroff's test	++	++	-	++
Hager's test	-	++	++	++
Wagner's test				
Test for Carbohydrates and glycosides				
Fehling test	-	-	-	-
Molish test	-	++	-	++
Benedict test				
Keller-Killiani test	-	++	-	-
Selivanoff test	-	-	-	-
Legel's test	-	-	-	-
Test for phenolic compounds and Tannins				
Dil. $FeCl_3$-test	-	-	-	++
Vanillin HCl test	-	-	-	++
Test for sterols / Triterpenoids				
Salkowaski test	-	++	-	-
Liberman Burchard's test	-	++	-	-
Test for Saponins				
Saponins Test				
Test for Proteins and Amino acids				
Millon's test	-	-	-	-
Ninhydrin test	-	-	-	-
Biuret test	-	-	++	-

(-) Absence, (++) Presence

Qualitative phytochemical constituents of *Ocimum gratissimum* (Linn.) leaves: All the extracts were screened for phytochemical constituents. Results showed the presence of alkaloids, triterpenoids, carbohydrates and glycosides in chloroform extract; acetone extract showed the presence of alkaloids, proteins and amino acids, while methanol extract was rich in alkaloids, carbohydrate, glycosides and phenolic compounds (Table- 2).

Conclusion

From extensive literature survey, it was found that *A. humile* and *O. gratissimum* (Linn.) possess different pharmacological activities that have been reported. The Allium Genus includes approximately 500 species that most widely used and have been employed for centuries for the pungency and flavouring value, and for their medicinal properties. The scientific community is increasingly getting interested in the study of pharmalogical properties of Allium plants and their chemical constituents particularly with regard to their ability to prevent various diseases[12, 13] whereas various leaf extracts of *O. gratissimum* (Linn.) showed antidiabetic, anti-diarrheal, anti-fertility, hepatoprotective, analgesic activity aphrodisiac, immuno stimulatory, Antitumor, anti-mutagenic and anti-cancer effects have been reported in in-vitro and *in-vivo* experiments[14-15]. The widespread plant is known for its chemopreventive, anticarcinogenic, free radical scavenging effect and used as traditional herb in European and Asian countries since ancient times[16]. *Allium humile* and *Ocimum gratissimum* (Linn.) leaves are widely used in folklore medicine for treatment of various diseases in the different regions of Himalaya.

In present study, the plant materials were subjected to soxhlet extraction using various solvents in an order of increasing polarity viz., petroleum ether, chloroform, acetone and methanol. The extracts thus obtained were concentrated and used for further evaluation. Qualitative phytochemical analysis of different extract was carried out. Methanol extract of *A. humile* was rich in alkaloids, saponins, carbohydrate, glycosides, proteins and amino acids and chloroform extract of *O. gratissimum* (Linn.) gave test for alkaloids, triterpenoids, carbohydrates and glycosides.

Acknowledgement

The authors are grateful to Director and management of SBSPGI, Balawala, Dehradun, India for providing the necessary facilities during this experimental study.

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Antioxidant Effect and Antimicrobial Activity of *Cassia auriculata*

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Abstract- *Cassia auriculata* (Fabaceae) commonly known as "Tanner's cassia"is a shrub with large bright yellow flowers found growing wild in central and western India and cultivated in other areas of the country. It is widely used in Indian folk medicines for the treatment of various diseases like diabetes, rheumatism and conjunctivitis. Free radicals are generated inside the body due to various physiological and environmental factors which cause various ill effects on the body including ageing and cancer. The objective of, the present study is to discover a new entity which can inhibit the generation of these free radicals and also its anti-microbial activity. Consuming antioxidant supplements would help on maintaining immune system in the best conditions possible. It has been found that when large numbers of antioxidants are mixed together in small doses, they can help in protecting against severe diseases like cancer and heart diseases.

Introduction

Antioxidants are substances that reduce oxidative damage in cells caused by free radicals.[1] Medicinal properties of plants are due to presence of definite chemical constituents in them. In many cases these physiologically active constituents of medicinal plants give a clue for the preparation of synthetic drugs of greater potency. With the development in technology and recent research, it has been proved that certain non-nutritive chemicals in plants such as Terpenoids and Flavanoids possess antioxidant properties [2,3]. Antioxidants are a type of complex compounds found in our diet and act as protective shield for our body against certain disastrous diseases and strengthen the immune system of our body[4]. Medicinal plants contain various compounds like carotenoids, vitamins, terpenes, flavanoids and phytosterols etc. that possess antioxidative components which prevent from free radical damage and premature ageing.[5-7]Some of the important medicinal plants having characteristic antioxidant properties are tabulated here in alphabetical order with their common name, family, part of the plant used and their active chemical constituents[8-11]. *Cassia auriculata* (Fabaceae) commonly known as "Tanner's cassia" is a shrub with large bright yellow flowers found growing wild in central and western India and cultivated in other areas of the country. It is widely used in Indian folk medicines for the treatment of various diseases like diabetes, rheumatism and conjunctivitis.

Material and Methods

This study is comprised of mainly two activities listed below as separate headings for which different tests are carried out.

In-vitro Antioxidant Activity[1]

DPPH radical scavenging method: The different concentrations of extracts, the compound and standards were added to DPPH in methanolic solution (100 µM) in a 96-well microtitre plate. After incubation at 37^0C for 20 min, the absorbance of each solution was determined at 490 nm using ELISA reader.

Scavenging of super oxide radical by alkaline DMSO method: The reaction mixture is prepared containing NBT (1 mg/ml solution in DMSO) and alkaline DMSO (1ml DMSO containing, 5mM NaOH in 0.1ml water) and to its different concentrations of the extracts and standards were added and the absorbance was measured at 560 nm.

Nitric oxide radical inhibition assay: The reaction mixture containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml) and the extracts, the compound and standard solutions (1 ml) were incubated at 25 ^{0}C for 150 min. After incubation, 0.5ml of the reaction mixture was removed and 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1ml of NEDD was added, mixed and allowed to stand for another 30 min in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions in a 96-well microtitre plate using ELISA reader.

Scavenging of ABTS radical caution: To various concentrations of the extracts, the compound or standard, distilled DMSO and ABTS solutions were added and incubated for 20 min. Absorbance was measured at 734 nm.

Anti-microbial Activity[3]

Agar Diffusion Method- Media prepared according to the manufacturers instruction (for each organism). Wells were prepared and 40 µl of the different

concentrations of the extracts were introduced into the well and was incubated at 37ÚC for bacteria for 48 hrs. Anti-microbial activity was determined by measuring the zone of inhibition.

Antioxidant Activity

Assay	IC50 value (µg/ml)
DPPH	24
NBT	110
Nitric Oxide	>1000
ABTS	>1000

Anti-microbial Activity

Organism/ Conc. (µg/ml)	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*B. cereus*	*B. subtilis*	*S. aureus*
200	17	-	17	18.5	14	19.5
100	14	-	-	13	12	16.5
50	-	-	-	15	-	12.5
25	-	-	-	13	-	11.5

The crude extract of *Cassia auriculata* showed significant antioxidant activity in DPPH and NBT. It also showed significant anti-microbial activity.

Conclusion

From these studies it can be concluded that the components in the crude extract of *Cassia auriculata* contains active components responsible for antimicrobial and antioxidant activity. Further isolation of these active components from the fraction would lead to the identification of the molecule.

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Antimicrobial Activity of *Cleome viscose (seed)*

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Abstract- The present study was aimed at detecting and evaluating antimicrobial activities of *Cleome viscosa* known for their medicinal properties in folk medicine. Methanol and acetone extracts of seeds show good activity against some bacterial strains such as *proteus vulgaris, bacillus cereus, pseudomonas aeruginosa, klebsella pneumonia*. Methanol extracts showed maximum antibacterial activity in comparison to other extracts. Methanol extract also shows good antifungal activity against *Aspergillus niger.* It showed maximum antifungal activity in comparison to other extracts.

Key words: *Cleome viscosa*, Antimicrobial activity.

Introduction

Cleome viscosa is a plant belonging to family Capparaceae. It is a weed distributed throughout the tropics of the world and the plains of india. The plant is an annual; sticky herb with a strong penetrating odour. It is known as Hurhur (Hindi), hurhuria (Bengali), Nayikkadugu (Tamil) in indian traditional medicine[1-2]. Leaves are digitately compound with 3-5 leaflets. Fruit 30-75 mm long, 3-5 mm broad, linear-oblong, erect, obliquely striated, tapering at both ends, glandular-pubescent, slender; style 2-5 mm long; seeds many, 1-1.4 mm in diam., glabrous with longitudinal striations and transverse ridges, dark brown. *Cleome viscosa* is highly effective in wide spectrum of disease and reported to posses antidiarrhoeal, analgesic, pharmacological, antimicrobial properties including in vitro Helicobacter pylori and wound healing activity[3].

Material and Methods

Collection of seed: Leaves of Cleome viscosa were collected from area around Tilak nagar, Delhi during the month of Oct to Dec. The collected plant material was washed with water to remove mud and other undesirable material and then dried under shade.

Extraction of seed: The collected plant material was washed with water to remove other undesirable materials and dried under shade. The air-dried seeds (300 gm) of *Cleome viscosa* were crushed. The crushed seeds extracted with methanol at room temperature. The extract was evaporated till dryness to obtain residue. These extracts were concentrated under reduced pressure. The extract was used for antimicrobial activity.

Anti-microbial activity: The **anti-microbial activity** of seed of *Cleome viscose* was carried out. The seed extracts were screened for anti bacterial and anti fungal activities.

Anti bacterial activity of seed extract: In this study, the anti bacterial activity was studied against the micro organism and the bacterial cultures used in the study were:

1) *Pseudomonas*
2) *Klebsiella species*
3) *B.cereus*
4) *Protius*

These bacterial cultures were maintained on nutrient agar slants at first being incubated at 37°c for about 18-24 hours and then stored at 4°c as stock for anti bacterial activity. Fresh cultures were obtained by transferring a loop full of cultures into nutrient broth and then incubated at 37°c overnight. To test anti bacterial activity, the well diffusion method was used.

Culture media preparation: The microbiological media prepared as per standard instructions provided by the HI-Media Laboratories, Mumbai. The media used for anti-bacterial activity were Muller- Hinton Agar (MHA) and Nutrient broth (NB). They were prepared and sterilized at 121°C at 15 psi for 15-30 minutes in autoclave.

Plate preparations: 25 ml of pre autoclaved Muller-Hinton agar (MHA) was poured into 90 mm diameter pre sterilized petri-plates. These petri-plates were allowed to solidify at room temperature.

Well Diffusion method: After the plated solidified, the freshly prepared microbial growth culture suspension (about 20µl) was spread over the Muller – Hinton agar (MHA) media using L shaped sterilized glass spreader separately under the aseptic condition using laminar air flow. Then, well were made in each plate with the help of borer of 8 mm diameter. In these well, about 100µl of each seed extracts individually was loaded. This method depends upon the diffusion of seed extracts from hole through the solidified agar layer of petri-dish to such an extent that the growth of added micro organism is prevented entirely in a circular area or zone around the hole containing seed extract. Petri plates were incubated for overnight at 37°C ± 0.5°C in the incubator.

Inhibition Measurement of zone of inhibition: After incubation, the diameter of clear zone of incubation produced around the well or holes were measured in mm by ESR Tube and compared with standard drug.

Results and Discussion

Antimicrobial studies reveal that methanol and acetone extracts of seeds show good activity against some bacterial strains such as *proteus vulgaris, bacillus cereus, pseudomonas aeruginosa, klebsella pneumonia*. Methanol extracts showed maximum antibacterial activity in comparison to other extracts.All extracts showed antifungal activity against all bacterial culture at a concentration of 200 mg/ml.Methanol extract showed maximum antifungal activity in comparison to other extracts.

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Table-1 Antibacterial Activity of the Extract of *Cleome viscose* Seed

Test organism	Inhibition zone (mm)				
	Pt.ether	Chloroform	Acetone	Methanol	Standard drug (chloramphenicol)
Pseudomonas	-	8	9	15	22
Klebsiella species	-	4	12	14	20
B.cereus	-	6	10	17	22
Protius	-	5	13	16	19

Table- 2 Antifungal Activity of the Extract of *Cleome viscose Seed*

Test organism	Inhibition zone (mm)				
	Pt.ether	Chloroform	Acetone	Methanol	Standard drug (chloramphenicol)
Aspergillus niger	9	11	8	13	18

Physico-chemical Characteristics of *Diospyros peregrina* Fruits at Different Ripening Stages

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Abstract- *Diospyros* is a large genus of shrubs and trees comprising of 500 species distributed in the warmer regions. It belongs to the family Ebenaceae. About 41 species occur in India mostly on evergreen forests of Deccan, Assam, and Bengal; only few are found in North India. Ripening stage of this fruit affects physical parameters of fruit which ultimately affect the quality of processed products prepared from them. Hence, an experiment was undertaken to study the effect of different stages of ripening on physical parameters and chemical composition of *Diospyros* fruits. In this study, it was observed that the ripening stages of the fruit cause variations in volume and weight of fruit, its pulp specific gravity as well as in its amount of reducing sugar, tannin, ascorbic acid. These phsico-chemical variations in fruit may be taken as maturity indices to judge its ripening stages.

Introduction

The fruits of *Diospyros peregrina* fall to ground from June to July onwards and, under favorable conditions, the seeds germinate during rainy season. Its fruit is spherical berry with a leathery rind, containing 4-8 seeds embedded in a viscid glutinous pulp. It is yellow when ripe and is covered with a rusty easily detachable scurfiness. A good tree produces about 4000 fruits in a season. Its fully ripe fruits have a mawkish sweet taste and are edible. Unripe fruits are rich in tannin and are employed for tanning hides and dyeing cloth. The fruit and the stem bark possess astringent properties. The unripe fruit is acrid, bitter and oleaginous. An infusion of the fruit is used as gargle in aphthae and sore throat. The juice forms a useful application for wounds and ulcers.

Its fruits are used for making jams, jellies, osmodehydrated slices and squash (Reddy, 1959). Products like sweet chutney, dried pieces, milk shake, nectar, blended drinks, pickle, preserve and candy can also be prepared with good sensory quality (Sawant, 1989). Even wine can be prepared from *Diospyros* fruit (Gautam and Chundawat, 1998).

The present investigation was conducted at the Department of Chemistry, Forest Research Institute, Dehradun (India) during the years 2009 to 2012. Experiments at Dehradun have shown that the viability of seeds, which is high when fresh, suffers during storage. Collection of fruit at different stages during its ripening was done and the fruits of different stages during fruit ripening were stored and kept into deep freeze in the laboratory at –20°C temperature. These fruits of different stages of ripening were used for physico-analysis.

Material and Methods

The fruits of *Diospyros peregrina* used in the study were collected at four different stages viz., immature green, mature green, colour initiation and fully ripe from the Forest Research Institute garden, Dehradun, India. The experiment was conducted by following complete randomized design with 4 levels of ripening stages with 5 replications. To study the physical characteristics, 10 *Diospyros* fruits from each ripening stage were randomly selected and examined individually for various physical parameters. The average of 10 fruits has been reported for each physical parameter.

The weights of fruit, pulp, skin and seed were measured by Electronic balance at different stages of fruit ripening. Volume of fruit was measured by measuring cylinder with reference to water, length and diameter, pH of fruit by vernier calipers and pH indicator at different stages of fruit ripening.

Results and Discussion

It is seen from Table- 1 that all the ripening stages of *Diospyros* fruit had significant influence on physical parameters of *Diospyros* fruit. Weight of fruit and seed, volume of fruit, length and diameter of fruit showed increasing trend from immature stage to fully ripe stage during ripening of *Diospyros* fruit and significantly decreasing trend of pulp: seed ratio. Significantly, increasing trend of weight of pulp, weight of skin and specific gravity at different stages of fruit ripening in Diospyros fruit has been shown. This increasing trend obtained during ripening of *Diospyros* fruit may be attributed to gain of moisture due to respiration and transpiration during ripening process. The present findings of decreasing trends in weight, volume, length and diameter of fruit are supported by Pawar (1988) in karonda fruit and Honde (1995) in sapota for juice recovery. Minimum weight of skin observed at mature stage (1.19g) was the impact of loss of maximum moisture from skin. However, increase in specific gravity of fruits from 1.07 (immature stage) to 1.30 (fully ripe stage) during

ripening indicated that the increase in weight of fruit was more than the corresponding increase in its volume. But, specific gravity of *Diospyros* fruit at different stages of ripening did not show significant difference. Similar observations have been reported by Joshi *et al.* (1986) for karonda fruits. Weight of pulp and pulp: seed ratio of *Diospyros* fruit at different stages of ripening did not show significant difference. Decrease in pulp: seed ratio observed during ripening of *Diospyros* fruit may be due to more increase in weight of pulp as compared to seed. Observation analogous to this finding was reported by Raut (1999) in sapota fruits. The colour of *Diospyros* fruit changed from dark green (immature stage) to yellow (fully ripe stage) during ripening. Identical observations during ripening were also reported by Suryanarayana and Goud (1984) and Raut (1999) in sapota.

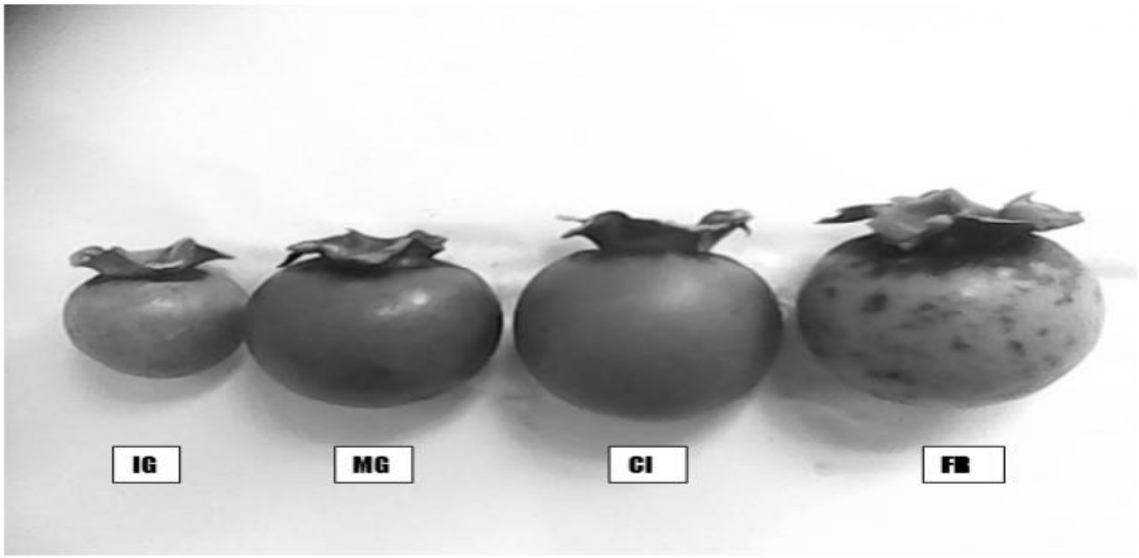

Figure- 1 Immature Green (IG), Mature Green (MG), Colour Initiation (CI) and Fully Ripe (FR) fruits of *Diospyros peregrina*.

Table 1. Effect of different stages of ripening on physical parameters of *Diospyros peregrina* fruits

Ripening Stage	Weight of fruit (g)	Weight of pulp (g)	Weight of skin (g)	Weight of seed (g)	Volume of fruit (ml)	Specific gravity	Length of fruit (cm)	Diameter of fruit (cm)	Colour	pH	Pulp: Seed ratio
1st	16.08	5.90	1.23	1.23	15	1.07	4.5	5.2	Dark green	6.27	4.78
2nd	28.84	16.42	1.19	3.74	25	1.15	5.0	5.5	Light green	5.91	4.38
3rd	32.23	14.65	1.53	5.82	30	1.07	5.2	5.8	Yellowish green	6.51	2.51
4th	52.03	28.52	3.94	8.60	40	1.30	6.4	7.8	Yellow	6.14	3.31

1st – Immature green stage, 2nd – Mature green stage, 3rd - Colour initiation stage, 4th - Fully ripe stage

The pH in immature stage (6.27), mature stage (5.91), colour initiation (6.51) and fully ripe (6.14) found during ripening of *Diospyros* fruit may be attributed to the decrease in acidity during ripening. Results of the present study are supported by Paralkar (1985) in sapota and Pawar (1988) in karonda. The pH values were significant at different stages during fruit ripening in *Diospyros* fruit.

It is observed from Table- 2 that all the chemical parameters studied showed significant difference with respect to ripening stages of fruit except moisture content of fruit. Total and reducing sugar content of *Diospyros* fruit at different stages of ripening increased significantly from immature green (13.20% and 7.80%) to colour initiation stage (18.23% and 10.09%) with a slight decline at fully ripe stage (19.40% and 9.98%). An increase in sugars during ripening process in *Diospyros* fruit may probably be due to accumulation of more sugars in the fruit due to hydrolysis of starch and slight decline at over ripe stage was due to utilization of sugars during respiration process. The results of this investigation are in agreement with the results obtained by Raut (1999) in sapota fruit.

Table- 2 Effect of different stages of ripening on chemical composition of *Diospyros peregrina* fruits

Ripening Stage	Total sugars (%)	Reducing sugars (%)	Titratable acidity (%)	Ascorbic acid (mg/100g)	Tannins (%)	Moisture (%)
1st	13.20	7.80	0.24	20.50	0.43	75.70
2nd	15.67	8.90	0.21	14.23	0.30	74.20
3rd	18.23	10.09	0.14	11.98	0.19	72.30
4th	19.40	9.98	0.11	7.28	0.14	69.70

1st – Immature green stage, 2nd – Mature green stage, 3rd - Colour initiation stage, 4th - Fully ripe stage

Ascorbic acid content of *Diospyros* fruit declined throughout the ripening process from 20.50 mg/100g (immature green stage) to 7.28 mg/100g (fully ripe stage) due to oxidative destruction of ascorbic acid by enzymes, mainly ascorbic acid oxidase, during ripening (Hulme, 1970). Identical observations during ripening were also reported by Suryanarayana and Goud (1984) and Raut (1999) in sapota. Sharp decrease in tannins was observed during ripening of *Diospyros* fruit. This may be due to the fact that tannins are hydrolyzed into components like sugars, acids and other compounds during ripening and also due to its oxidation by polyphenol oxidase to form colour pigment. Similar results were reported by Sawant (1989) in sapota. The moisture content of *Diospyros* fruit decreased continuously from immature green (75.70%) to fully ripe stage (69.70%). However the results were nonsignificant. Decline in moisture of *Diospyros* fruit during ripening could be attributed to the loss of moisture through respiration and transpiration. Similar findings were also reported by Raut (1999) in sapota fruits cv. Kalipatti.

Conclusion

From the present study, it is concluded that physical parameters *viz.* weight of fruit, pulp, skin and seed, volume of fruit, specific gravity, length and diameter of fruit, fruit colour, and pulp: seed ratio and chemical parameters *viz.* total and reducing sugars, titratable acidity, ascorbic acid, tannins and moisture of *Diospyros* fruits could be considered as maturity indices to judge the ripening stages of *Diospyros* fruit for various value added products preparation.

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Forced Degradation Studies for Drug Substances and Drug Products- Scientific and Regulatory Considerations-Review

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Abstract- Forced degradation is the process of subjecting drug compounds to extreme chemical and environmental conditions to determine product breakdown levels and preliminary degradation kinetics, and to identify potential degradation products. They are used to facilitate the development of analytical methodology, to gain a better understanding of active pharmaceutical ingredient (API) and drug product (DP) stability, and to provide information about degradation pathways and degradation products. It is particularly useful when little information is available about potential degradation products. In addition to develop stability-indicating analytical methods, these studies also provide information about the degradation pathways and degradation products that could form during storage, transportation. Forced degradation studies may help facilitate pharmaceutical development in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product. This publication provides information about regulatory needs and scientific guidance to perform forced degradation.

Introduction

Forced degradation studies are also known as stress testing, stress studies, stress decomposition studies, forced decomposition studies, etc. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used[1]. But these guidelines are very general in conduct of forced degradation and do not provide details about the practical approach towards stress testing.

Knowledge of the stability of molecule helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life, which is essential for regulatory documentation. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule[2].

Although forced degradation studies are a regulatory requirement and scientific necessity during drug development, it is not considered as a requirement for formal stability program.

Overview of regulatory guidance: According to the available guidance, forced degradation studies are carried out for the following reasons:

1. Development and validation of stability-indicating methodology.
2. Determination of degradation pathways of drug substances and drug products.
3. Discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances (e.g., excipients).
4. Structure elucidation of degradation products.
5. Determination of the intrinsic stability of a drug substance molecule. Degradation studies have several defining characteristics.
6. They are carried out in solution and/or the solid state.

7. Involve conditions more severe than accelerated testing (e.g., 40 C; 75% relative humidity; in excess of ICH light conditions; high and low pH, oxidation, etc.) (1, 2).
8. Are typically carried out on one batch of material (1, 2).
9. Include conditions that analyze thermolytic, hydrolytic, oxidative, and photolytic degradation mechanisms in the drug substance and drug product (as appropriate) (1, 2).
10. Is not part of the formal stability program.

FDA perspectives and scientific considerations: Ragine Maheswaran provided a clear perspective on FDA regarding the scientific considerations with respect to forced degradation studies. If the substance does not show any degradation under any of the stress conditions then the Stress studies shall be repeated to obtain adequate degradation. If degradation is not achievable, rationale shall be provided. The conditions employed for stress study are too harsh and that most of the drug substance has degraded. The stress studies using milder conditions or shorter exposure time to generate relevant degradation products. Stressed samples shall be performed as per the assay method conditions. For the related substances method to be stability indicating, the stressed samples should be analyzed using related substances method conditions. The attempts shall be made to ensure that all the impurities including the degradation products of the unstressed and the stressed samples are captured by the final analytical method. Summary of the amount of degradation products (known and unknown) in the stressed samples shall be provided. The purity determinations shall be performed as per the established software. Mass imbalance of the stressed samples shall be justified. The degradation products shall be identified that are formed due to drug-excipients interactions. Photo stability studies shall be determined whether the drug product is very sensitive to light or not. This shall be documented in the analytical method, manufacturing process, product handling and etc[3].

Forced degradation in QbD paradigm: A systematic process of manufacturing quality drug products that meet the predefined targets for the critical quality attributes (CQA) necessitates the use of knowledge obtained in forced degradation studies.

A well-designed, forced degradation study is indispensable for analytical method development in a QbD paradigm. It helps to establish the specificity of a stability indicating method and to predict potential degradation products that could form during formal stability studies. Incorporating all potential impurities in the analytical method and establishing the peak purity of the peaks of interest helps to avoid unnecessary method re-development and revalidation.

Knowledge of chemical behavior of drug substances under various stress conditions can also provide useful information regarding the selection of the excipients for formulation development. Excipients compatibility is an integral part of understanding potential formulation interactions during product development and is a key part of product understanding. Degradation products due to drug-excipient interaction or drug-drug interaction in combination products can be examined by stressing samples of drug substance, drug product, and placebo separately and comparing the impurity profiles. Information obtained regarding drug-related peaks and non-drug-related peaks can be used in the selection and development of more stable formulations. For instance, if a drug substance is labile to oxidation, addition of an antioxidant may be considered for the formulation. For drug substances that are labile to acid or undergo stereo-chemical conversion in acidic medium, delayed-release formulations may be necessary. Acid/base hydrolysis testing can also provide useful insight in the formulation of drug products that are liquids or suspensions.

Knowledge gained in forced degradation studies can facilitate improvements in the manufacturing process. If a photostability study shows a drug substance to be photolabile, caution should be taken during the manufacturing process of the drug product. Useful information regarding process development (e.g., wet versus dry processing, temperature selection)

can be obtained from thermal stress testing of drug substance and drug product[3].

In addition to develop stability indicating methods, forced degradation studies provide information for degradation pathways and degradation products that could form during storage and transportation. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product.

Degradation Conditions: Typical stress tests include four main degradation mechanisms: heat, hydrolytic, oxidative, and photolytic degradation. Selecting suitable reagents such as the concentration of acid, base, or oxidizing agent and varying the conditions (e.g., temperature) and length of exposure can achieve the preferred level of degradation. Over-stressing a sample may lead to the formation of secondary degradents that would not be seen in formal shelf-life stability studies and under-stressing may not serve the purpose of stress testing. Therefore, it is necessary to control the degradation to a desired level. A generic approach for stress testing has been proposed to achieve purposeful degradation that is predictive of long-term and accelerated storage conditions[5]. The generally recommended degradation varies between 5-20% degradation[5-8]. This range covers the generally permissible 10% degradation for small molecule pharmaceutical drug products, for which the stability limit is 90%-110% of the label claim. Although there are references in the literature that mention a wider recommended range (e.g., 10-30%), the more extreme stress conditions often provide data that are confounded with secondary degradation products.

Table Conditions generally used for forced degradation studies

Degradation type	Experimental conditions	Storage conditions	Sampling time (days)
Hydrolysis	Control API (no acid or base)	40°C, 60°C	1,3,5
	0.1M HCl	40°C, 60°C	1,3,5
	0.1 M NaOH	40°C, 60°C	1,3,5
	Acid control (no API)	40°C, 60°C	1,3,5
	Base control (no API)	40°C, 60°C	1,3,5
	pH: 2,4,6,8	40°C, 60°C	1,3,5
Oxidation	3%H_2O_2	25°C, 60°C	1,3,5
	Peroxide control	25°C, 60°C	1,3,5
	Azobisisobutyronitrile (AIBN)	40°C, 60°C	1,3,5
	AIBN control	40°C, 60°C	1,3,5
Photolytic	Light 1 × ICH	NA	1,3,5
	Light 3 × ICH	NA	1,3,5
	Light control	NA	1,3,5
Thermal	Heat chamber	60°C	1,3,5
	Heat chamber	60°C/75% RH	1,3,5
	Heat chamber	80°C	1,3,5
	Heat chamber	80°C/75% RH	1,3,5
	Heat control	Room temp.	1,3,5

Selection of experimental conditions: There are many examples in the literature of experimental conditions it not possible to identify a generic set of conditions for a forced degradation study. For an early phase molecule, using a set of normal conditions by first intention makes sense since very little may be known about the intrinsic stability. If early stability data are available which suggest the molecule is labile at a particular condition (e.g., high pH), the conditions can be modified to take into account the instability (e.g., reduced temperature or time of study). Once a set of conditions have been found, they may be repeated whenever a new stability-indicating method is required during development. Therefore, for later-phase molecules, the forced degradation conditions are defined by the earlier work. By reprocess the same forced degradation conditions throughout development a consistent approach is maintained[4]. Some conditions mostly used for forced degradation studies are presented in Table-1.

1. Hydrolytic condition: Hydrolysis is one of the most common degradation chemical reactions over wide range of pH. Hydrolysis is a solvolytic process in which drug reacts with water to yield breakdown products of different chemical compositions. Water either as a solvent or as moisture in the air comes in contact with pharmaceutical dosage forms is responsible for degradation most of the drugs. For example, aspirin

combines with water and hydrolyzed to salicylic acid and acetic acid[9-10]. Hydrolytic study under acidic and basic condition involves catalyzation of ionisable functional groups present in the molecule. HCl and NaOH are employed for generating acidic and basic stress samples, respectively[11]. The hydrolytic degradation of a new drug in acidic and alkaline condition can be studied by refluxing the drug in 0.1 N HCl / 0.1 N NaOH. If reasonable degradation is seen, testing can be stopped at this point. However in case no degradation is seen under these conditions the drug should be refluxed in acid/alkali of higher strength and for longer duration of time. Alternatively if total degradation is seen after subjecting the drugs to initial condition, acid/alkali strength can be decreased with decrease in reaction temperature. In general temperature and pH are the major determinant in stability of the drug prone to hydrolytic decomposition. Hydrolysis of most of the drugs is dependent upon the relative concentration of hydronium and hydroxyl ions. Hence pH at which each drug is optimaly stable can be determined.

2. Oxidation conditions: Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen per oxide at neutral pH and room temperature for seven days or upto a maximum 20% degradation could potentially generate relevant degradation products[12]. The mechanism of oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulphides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulphones and sulphoxide[13]. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α–positions with respect to hetro atom is susceptible to oxidation to form hydroperoxides, hydroxide or ketone[14, 15].

3. Photo degradation: According to ICH Q1B guideline for photo degradation, samples should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter with spectral distribution of 320-400nm to allow direct comparisons to be made between the drug substance and drug product. Samples may be exposed side-by-side with a validated chemical actinometric system to ensure the specified light exposure is obtained, or for the appropriate duration of time when conditions have been monitored using calibrated radiometers/ lux meters[16]. The photolytic degradation can occur through non-oxidative or oxidative photolytic reaction. The nonoxidative photolytic reaction include isomerization, dimerization, cyclization, rearrangements, decarboxylation and hemolytic cleavage of X-C hetero bonds, N-alkyl bond (dealkylation and deamination), SO_2- C bonds etc and while oxidative photolytic reaction occur through either singlet oxygen ($^1O_2$) or triplet oxygen ($^3O_2$) mechanism. The singlet oxygen reacts with the unsaturated bonds, such as alkenes, dienes, polynuclear aromatic hydrocarbon to form photoxidative degradation products whereas triplet oxygen react with free radical of the drug molecule, which than react with a triplet oxygen molecule to form peroxide. Hence, light can also act as a catalyst to oxidation reactions[17, 18].

4. Thermal condition: In general, rate of a reaction increase with increase in temperature. Hence, the drugs are susceptible to degradation at higher temperature. Many APIs are sensitive to heat or tropical temperatures. For example, vitamins, peptides, etc. Thermal degradation involves different reactions like pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization. Effect of temperature on thermal degradation of a substance is studied through Arrhenius equation:

$$K = Ae^{-Ea/RT}$$

Where k is specific reaction rate, A is frequency factor, Ea is energy of activation, R is gas constant (1.987 cal/ deg mole) and T is absolute temperature.

Thermal degradation study is carried out at 40°C to 80°C. The most widely accepted temperature is 70°C at low and high humidity for 1-2 months. High temperature (>80°C) may not produce predictive degradation pathway. The use of high-temperatures in predictive degradation studies assumes that the drug molecule will follow the same pathway of decomposition at all temperatures. This assumption may not hold true for all drug molecules, and therefore great care must be taken in using the extreme temperatures easily accessible in a sealed-vessel microwave experiment for predictive degradation studies[19].

Conclusion

Forced degradation is important part of the drug development process as it provides knowledge about the degradation chemistry of drug substances and drug products. This knowledge is used primarily to develop stability- indicating analytical methods but also useful for other purposes such as formulation development, packaging development and the design of the official stability studies. As there is no formal regulatory guidance for forced degradation, it is recommended to use appropriate conditions to achieve 5-20% degradation.

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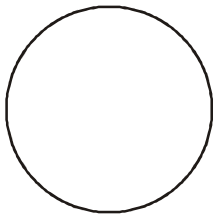
Mango *(* *Mangifera indica*) *shown* on the cover page- its Tree, Fruits, Flowers, Leaves and Bark.



Aam, Amra *(Mangifera indica)* is a species of mango in the Anacardiaceae family.

Introduction

Mango is a tropical and subtropical fruit crop grown in India over an area of 2.31 million hectares with production of 15.03 million tonnes (2009-10). Apart from as a delicious fruit its all parts (Unripe fruits, leaves, barks and flowers) are medicinally important.

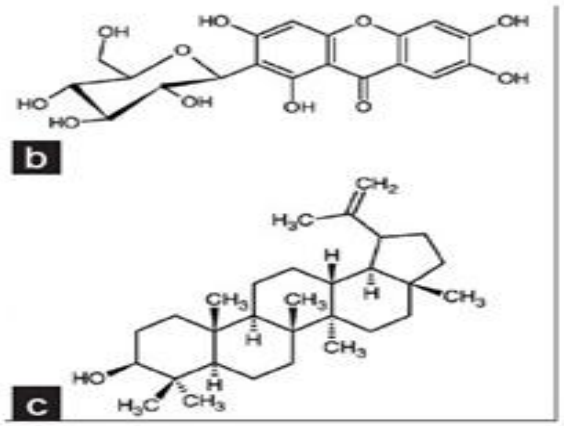
Fruit: Mango is the "king of fruits", it has strong aroma, delicious taste, and high nutritive value. Mango fruit are rich in pre-biotic dietary fiber, vitamins (Vitamin-A, vitamin-B6 (pyridoxine), vitamin-C and vitamin-E, Vitamin B-6), minerals like copper, and *poly-phenolic flavonoid* antioxidant compounds. These protect against colon, breast, leukemia and prostate cancers. Unripe green mangos are acidic, acrid, digestive and carminative and widely used in pickles, Panah, jam (marmalade), and chutney.

Bark: The bark is astringent, acrid, refrigerant, styptic, anti syphilitic, vulnerary, anti emetic, anti inflammatory and constipating.

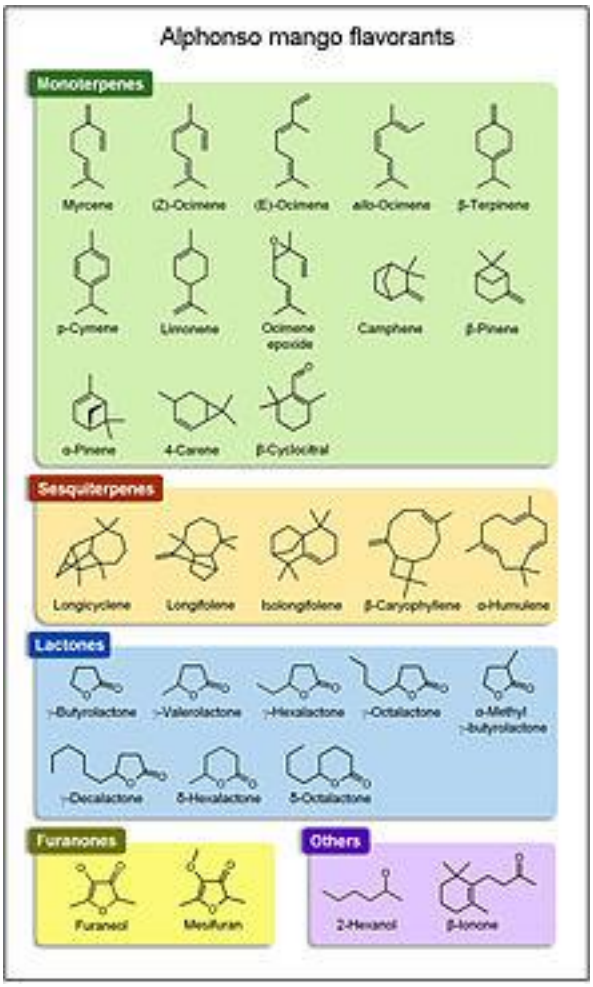
Leaves: The leaves are useful in vitiated conditions of cough and pitta, hiccup, hyperdipsia, burning sensation, hemorrhages, wounds, ulcers, diarrhoea, dysentery. The smoke from burning leaves is inhaled for relief in hiccup and throat diseases.

Flowers: The flowers are astringent, refrigerant, styptic, vulnerary, constipating and haematinic.

Stone: The seed kernel in rich source of protein (8.5%) and gallic acid. It also used in many digestive Choorans



Anti-diabetic constituents of Mango. (b) mangiferin, (c) lupeol


Alphonso mango flavorants
Monoterpenes
Myrcene
(Z)-Ocimene
(E)-Ocimene
allo-Ocimene
β-Terpinene
p-Cymene
Limonene
Ocimene epoxide
Camphene
β-Pinene
α-Pinene
4-Carene
β-Cyclocitral
Sesquiterpenes
Longicyclene
Longifolene
Isolongifolene
β-Caryophyllene
α-Humulene
Lactones
γ-Butyrolactone
γ-Valerolactone
γ-Hexalactone
γ-Octalactone
α-Methyl γ-butyrolactone
γ-Decalactone
δ-Hexalactone
δ-Octalactone
Furanones
Furaneol
Mesifuran
Others
2-Hexanol
β-Ionone

Forth Coming Events

1- 19th Annual Green Chemistry & Engineering Conference
Dates: July 14-16, 2015
Location: North Bethesda, Maryland, USA
Weblink: http://www.acs.org/content/acs/en/meetings/greenchemistryconferences.html

2- International Conference on Renewable Energy and Sustainable Environment (RESE – 2015) 3rd to 5th August 2015 Pollachi, Tamilnadu, India Website: http://www.drmcet.ac.in/conference

3- 2nd International Conference and Exhibition on Biotechnology Conference 3rd to 4th August 2015 Hyderabad, Telangana, India; Website: http://www.brightice.org/index.php?option=com_content&view=article&id=6&Itemid=109
International Conference and Expo on Pharmaceutical Sciences conference 3rd to 4th August 2015 Hyderabad, Andhra Pradesh, India Website: http://www.brightice.org/

4- Carbohydrate Processing Enzymes in Medicine and Biotechnology
Dates: 19 Aug 2015 - 21 Aug 2015
Location: University of St Andrews, United Kingdom
Weblink:http://www.biochemistry.org/Conferences/AllConferences/tabid/379/MeetingNo/SA169/view/Conference/Default.aspx

5- **Asia Labex 2015**
25-27 August 2015, New Delhi,India.
Venue : Pragati Maidan, New Delhi, India.
A prodigious international exhibition and conference on analytical, biotechnology, diagnostic, laboratory, research, testing & scientific instruments. The event will be the only show for entire lab customization where 2000 brands from around the world will be displayed. It will also provide best opportunity to "interact' with lab professionals and dealers from the neighbourhood and also "discuss" technicality of products during the seminar.
Organizers : Mr Pallav, Manager-International Sales, **M/s Fenza Exhibitions,** 21, 3rd Floor, Behind Samrat Restaurant, Outram Lane, Near G.T.B. Nagar Metro Station Gate No. 1, New Delhi- 110 009, India.
Phone: +91-11-4508 8781 / 8782. *Mobile*: +91-97111 82040.
Email:info@asialabex.com, asialabex@gmail.com
URL: www.asialabex.com

6- 4th International Conference on Applied Life Sciences (ICALS2015) , 15th to 17th September 2015 Mumbai, India ; Website: http://www.icals2015.com/

7- Indo-Global Summit and Expo On Healthcare 5th to 7th October 2015, New Delhi, India Website: http://healthcare.indoglobalsummit.com/

8- 14th International Conference on the Chemistry of Antibiotics and other Bioactive Compounds
Dates: 13 Oct 2015 '! 16 Oct 2015
Location: Galveston, United States
Weblink: http://www.icca-conference.com/

9- Pharmaceutical Summit and Expo, 8th to 10th October 2015, New Delhi, India Website: http://global.pharmaceuticalconferences.com/index.php

10- International Conference on Natural Products and Exhibition - INCONPE-2015, 8th to 10th October 2015, Namakkal, Tamil Nadu, India; Website: http://www.mypadacademia.com/inconpe2015

11- Global Conference on Renewable Energy (GCRE) , 19th to 21st October 2015 , Patna, Bihar, India Website: http://www.weentech.co.uk/gcre2015/

12- Current Scenario and Future Prospects of Biotechnology in Diverse Sectors (CSFPB-2015), 22nd to 24th October 2015, Sathyamangalam-

Erode, Tamil Nadu, India; Website: http://www.bitsathy.ac.in/csfpb2015/

13- 3rd International Conference and Exhibition on Pharmacognosy, Phytochemistry & Natural Products during October 26- 28, 2015, India, Hyderabad, India, Website:http://pharmacognosyphytochemistry-natural products.pharmaceuticalconferences.com/15.

14- 1st International Conference on Applied Chemistry – (ICAC 2015)
Date: 18–19 November 2015
This conference will cover the following areas:

- Water and Environmental Applications
- Oil, Gas and Energy
- Petrochemicals and Catalysis
- Nanotechnology and Advanced Materials
- Organic Synthesis, Natural Products and Medicinal Chemistry

Conference Venue
The conference will be held at King FAISAL CONVENTION CENTER- King Abdulaziz University – Jeddah, Saudi Arabia.
For Further information Please contact
Prof. Abdullah M. Asiri
E-Mail: aasiri2@kau.edu.sa

15- 6th International Conference and Exhibition on Pharmaceutics & Novel Drug Delivery Systems
Dates: March 07-09, 2016
Location: Madrid, Spain
Weblink: http://novel-drugdelivery-systems.pharmaceuticalconferences.com/

16- WBC2016 — 10th World Biomaterials Congress
Dates: 17 May 2016 '! 22 May 2016
Location: Montreal, Canada
Weblink: http://www.wbc2016.org

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