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RESEARCH ARTICLE

STUDIES ON ANTIBACTERIAL COMPOUNDS FROM METHANOLIC EXTRACT OF FRUIT (*KIMIA DATES*) OF *PHOENIX DACTYLIFERA* AND ITS APPLICATIONS

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Antiviral Activity.

ABSTRACT

The present study aims the evaluation of antimicrobial potential of methanol extract of Kimia dates and to identify potential natural sources for the synthesis of new drugs to address the growing antimicrobial resistance. The crude methanol extract of Kimia dates, Phoenix dactylifera was extracted and antibacterial activity was evaluated determined by paper disc method showed enterprising potential zone of inhibition of 17mm against Salmonella paratyphi B leading to the further investigation using agar cup method on various standard strains demonstrated excellent zone of inhibition (53mm) against *S. typhi.* MIC of E.coli and *S.aureus* found in the range of 0.195mg/ml and MBC in at 2.0mglml. Activity guided fraction was performed and found Ethyl acetate fraction exhibited promising activity with a zone of inhibition 38mm for *S.aureus* and 35mm for *E.coli.* Phytochemical analysis reveals the presence of glycoside, saponins, alkaloids, flavonoids and tannins. The potent ethyl acetate fraction was further subjected to characterization of bioactive compounds using HPTLC, followed by bioautography, CHNS, FTIR, LCMS and GCMS analysis revealing the presence of 12-Oleanen-3-yl acetate, (3α) (C32H5202) with probability-75% may be the bioactive compound in the Kimia ethyl acetate fraction, belonging to the class triterpenes, showing a wide range of antimicrobial, anti-diabetic, anti-amylase inhibitor, antioxidant, antibacterial, anti-inflammatory, antitumor activities. The Kimia dates ethyl acetate fraction showed the maximum antioxidant activity of 98.57µg/ml, MIC of antiviral activity is 6.25 mg/ml. The Fruit ethyl acetate fraction shows a strong ability to inhibit and reduce 96.48% infectivity of coli phage and completely prevented bacterial lysis at 30 minutes. The phage inactivation kinetics indicates 65.6% at 20 min of exposure. The fruit fraction showed a strong ability to inhibit the infectivity of coli phage and completely prevented bacterial lysis, which it is hoped will promote research into its potential as a novel antiviral agent against pathogenic human viruses.

Citation: Rajikrishna Ravishanker and Savanta V. Raut, 2016. "Studies on antibacterial compounds from Methanolic extract of fruit (*Kimia dates*) of *Phoenix dactylifera* and its applications", *International Journal of Current Research,* 8, (03), 28068-28078.

INTRODUCTION

Kimia Date Palm is of plant species of the family *Palmacea* that is its scientific name *Phoenix dactylifera*. Kimia Date is one of the most famous and delicious date fruit which is grown in the most Iran's south regions but it is more grown in southern Iranian city of Bam which has sweet taste, meaty and succulent flesh. The fruit of date palm (*Phoenix dactylifera* L.) is a basic dietary component of people living in the arid and semiarid regions in the world. It has a significant share of the economic and social role in livelihoods for the people of particular regions (El-Hadrami and Al-Khayri, 2012). Date fruit attains stepwise maturation stages that are internationally denominated by Arabic terms such as kimri (19 weeks after pollination: unripe, astringent, green and firm), khalal (29 weeks after pollination: partially-ripe, colored yellow or red depending on cultivar), rutab (30 weeks after pollination: fully-ripe, light-brown and soft) and tamar (31 weeks after pollination: dark- brown and soft, semidry or dry, highly sweet and storable) Kader and Hussein (2009). Chemically date fruit is composed of total sugars, dietary fibers, proteins, vitamins, fat, mineral contents and a very small starch content (Baliga *et al*., 2011; Vayalil, 2011), each of which may vary, depending on cultivar type, fruit maturation stage, soil type and agronomic practices (Al-Farsi *et al*., 2007b; Amira *et al*., 2011). In this context, it is pertinent to note that recent studies have indicated that the various parts of *Phoenix dactylifera* such as leaves, barks, pits, fruits and pollens have anticancer, antioxidant, hepatoprotective, antidiabetic, antihypertensive, anti-ulcerative, anti-inflammatory, antiproliferative, antimutagenic,

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antidiarrheal, antibacterial, antifungal and antiviral potential Mallhi 2014 Abedi 2012)

Experimental

Extraction of Kimia dates by Alade and Irobi's cold extraction Method (Perveen Kahkashan, 2012)

I. **Antibacterial Assay:-** (Al-daihan Sooda, 2012) Antimicrobial susceptibility test (AST) was used to determine the efficacy of potential antimicrobials from biological extracts. The crude methanol extracts of the Kimia dates were subjected to antimicrobial assay by:-

a) Paper disc diffusion method
b) Agar cup method/Agar well diffusion method

II. Minimum inhibitory concentration: (Mathur Rashmi, 2013)

a) Determination of Minimum Inhibitory Concentration (MIC)
b) Determination of minimum bactericidal Concentration (MBC)

III. Activity guided fractionation

Isolation of bioactive compounds from the Kimia dates, the sequential fractionation of crude methanol extracts with various organic solvents differing in their polarity, from highly polar to non-polar, and each obtained fraction subjected to bio assay. (Mathew *et al*., 2014)

a) **Phytochemical analysis:** (Stahl Egon, 2013; Wagner Hildebert and Sabine bladt, 1996) The qualitative tests to find the phytoconstituents present in the Kimia methanol fraction.
b) Chemical tests by tube method performed using the fractions extracted from activity guided fractionation are subjected to identify the constituents present in them.
c) Phytochemical analysis by HPTLC method for detection of class of compound.

IV. Characterization of Potent Kimia Fruit Ethyl Acetate Fractions: -

a) Detection of Bioactive compounds by Bioautography (Choma 2015),
b) Isolation and identification, of antimicrobial compounds includes HPTLC (Mahesh Attimarad 2011), CHNS, FTIR, LC-MS, and GC-MS analysis.

V. Determination of Antioxidant activity of Kimia Fruit Ethyl Acetate fractions by Phosphomolybdenum method (Ibrahim, 2012)

VI. Determination of Antiviral activity of Kimia Fruit Ethyl Acetate fractions

a) **Determination of minimum inhibitory concentration value:** The MIC of the test fruit extract was determined for test *coli phage* of *E. coli* as follows: The Microdilution method using 96 well microliter plates described by the National Committee for Clinical Laboratory Standards (NCCLS) was used. (Jassim Sabah, 2010)

b) Phage inactivation Assays: (Adams1959)

c) Determination of Phage Inhibition Kinetics: (Jassim sabah, 2010)

VII. Evaluation of antimicrobial activity of most potent fraction against some MDR pathogens

Agar cup diffusion method performed using sterile Mueller Hinton Agar, MDR strains and using standard antibiotics as control. (Sharifi Javad 2013)

RESULTS

Initial screening of anti-bacterial activity of the crude methanol extract of Kimia dates showed zone of inhibition of 17mm against *Salmonella paratyphi B* (Sabah *et al*., 2007; Ammar *et al*., 2009) adopting paper disc method showed enterprising potential leading to further investigation using agar cup method.

Fig. 1. Kimia Dates

Fig. 2. Alade and Irobi cold extraction

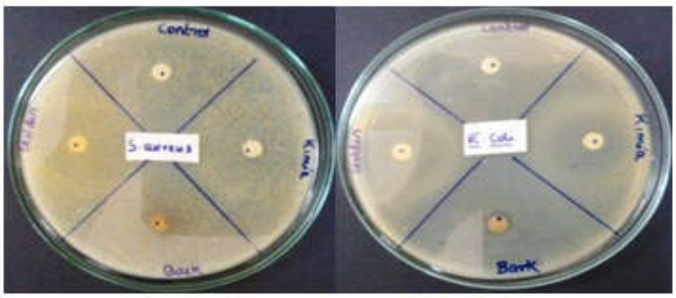

Fig. 3. Paper Disc Method (a) *S.aureus*, Plate (b) *E.coli*

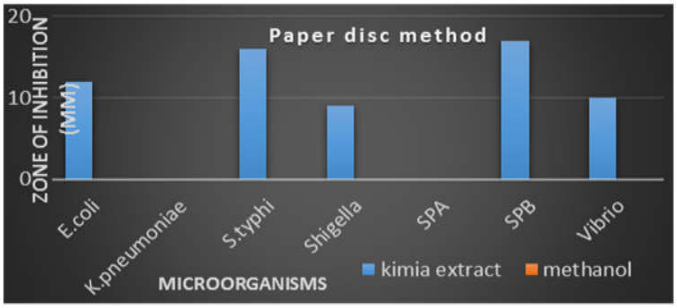

Fig. 4. Graphical representation of Antibacterial activity of crude methanol extract of Kimia dates by Paper Disc Method

Antibacterial property of the crude methanol Kimia extract was evaluated against 12 standard strains using agar cup method which demonstrated the inhibition of gram negative bacteria. The cold methanol crude extract of Kimia dates exhibits higher activity with zone of inhibition 53mm against *S.typhi* (Sooad Al-daihan and Ramesa Shafi Bhat, 2012).

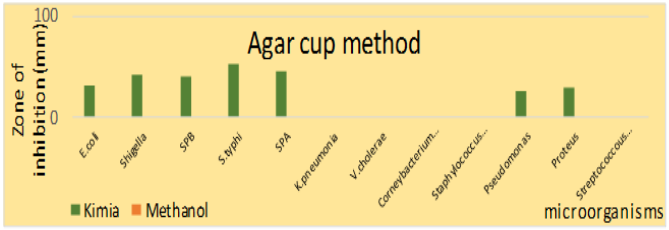

Fig. 5. Graphical representation of Antibacterial activity of crude methanol extract of Kimia dates by Agar cup Method

Fig. 6. *Proteus*

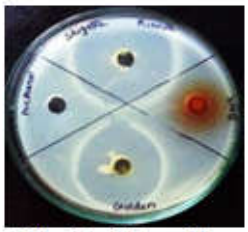

Fig.7. *Shigella*

Fig. 8. *SPB*

Fig.9. *E.coli*

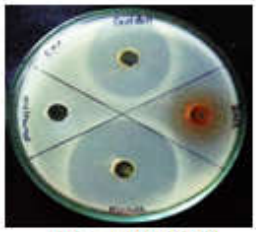

Fig. 10. *SPA*

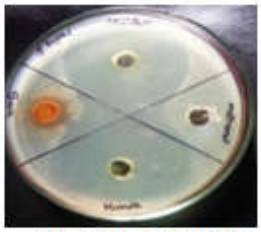

Fig.11. *S.typhi*

Figure: Result of Agar Cup Method

The crude extract of Kimia exhibited **MIC** values for *E.coli and S.aureus* was found in the range of 0.195 mg/ml. **MBC** for *E.coli* and *S.aureus* found in the range of 2.0 mg/ml, (earlier studies shows similar results (Kahkashan Perveen, Najat A. Bokhari, 2012).

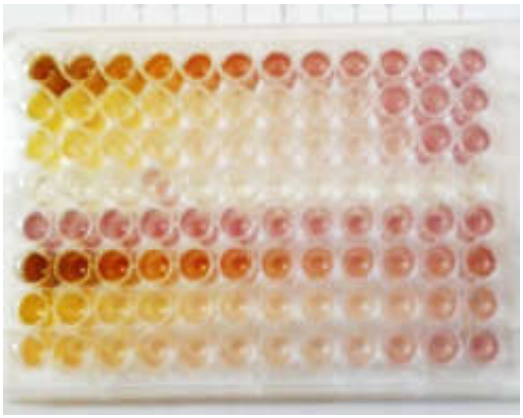

Fig. 12. MIC

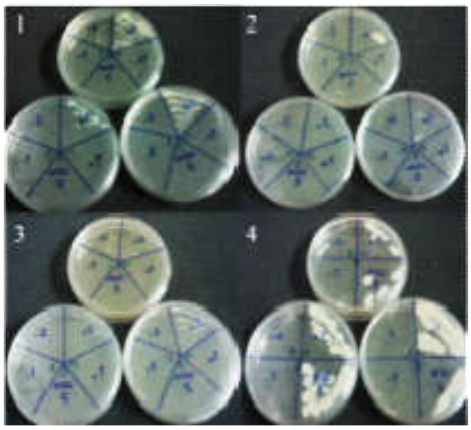

Fig. 13. MBC

Figure 14. Fractionation of kimia with different solvents varying in polarity

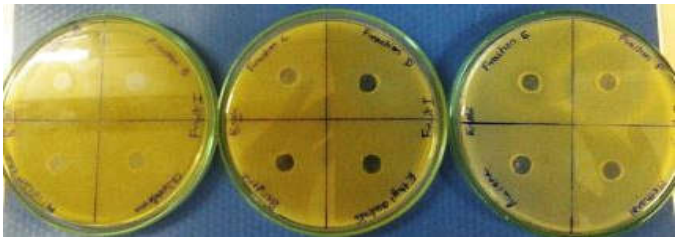

Fig. 15. AST of each fractions of Kimia on pathogens – *E.coli & S. aureus* and controls

Activity guided fractionation was performed on crude methanol fruit extract and the fractions are bio-assayed and the fruit ethyl acetate fraction showed maximum promising activity with a zone of inhibition of 38mm for *S.aureus* and 35mm for *E.coli* . *S. aureus* exhibited nil activity against crude methanol fruit extract and showed promising activity against semi purified ethyl acetate fraction (Cragg *et al,* 1996).

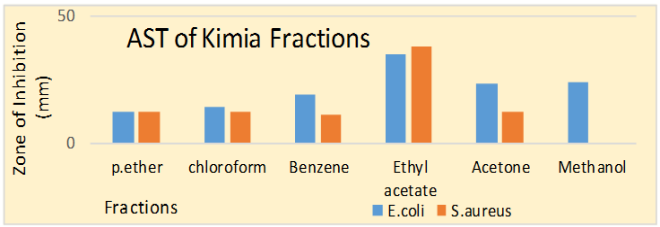

Fig. 16. Graphical representation of AST of each fractions of fruits on pathogens – *E.coli & S. aureus*

Phytochemical analysis (Chemical Tests by Tube Method) revealed the presence of phytoconstituents like glycoside, saponins, phytosterol, alkaloids, anthraquinone, flavonoids and trepenoids except tannins and coumarins (Sooad Al-daihan and Ramesa Shafi Bhat, 2012). Phytochemical analysis for detection of class of compounds by HPTLC profiled the levels of flavonoids, glycosides, alkaloids, saponins, and tannins. The Kimia ethyl acetate fractions was taken for further characterization of compounds and HPTLC was carried out. Based on the results of HPTLC for detection of class of phytochemical compound, the solvent system Toluene: Chloroform: Ethanol in the ratio 8:8:2 yielded better seperation.

From the HPTLC spectra of Fruit (Kimia date) Ethyl acetate fraction, 14 autogenerated peaks are showed at 254 nm The peak 5 that is with an Rf value of 0.24 showed sharp peak with an area of 10245.9 and an area% 20.74 at 254nm. More peaks are present at 254nm. There is only one autogenerated peaks showed at 366nm. Peak 9 with Rf value of 0.46 is a well defined sharp peak showing the highest area of 22863.7 with an area % of 32.83 at 540nm. Out of 14 peaks, peak 5 of Rf 0.24 showed maximum peak area 10245.9 and an area% 20.74 at 254nm. The kimia dates ethyl acetate fraction, 12 peaks were obtained from HPTLC spectra at 540nm, out of which peak 11 with Rf value 0.71 coincides with the corresponding Rf value 0.75 of Bioautography. (Stahl Egon, 2013) CHNS analysis of Kimia Ethyl acetate fraction the percentage as follows: Carbon (23.711%), Hydrogen (8.432)%, Nitrogen (2.060%), Sulphur(20.884) (Anoop Kumar Singh 2010). The FTIR Spectra of fruit ethyl acetate fraction spectra shows 16 peaks showing biomolecules revealing the presence of N-H-1^0,2^0, amines, amides (3382.98 cm^{-1}), 2929.70 cm^{-1} C-H stretching-alkanes, 1713.13 cm^{-1} C=O stretching-carbonyls and carboxylic acids, 1054.50 cm^{-1} C-N stretch-aliphatic amines. (*peak values interpretation based on standard table of characteristic IR absorptions) (Shib Shankar Dash 2013) The LC-MS spectra of Fruit I (Kimia dates) Ethyl acetate fraction shows 29 peaks and from spectrum 1A with maximum Base peak 218.7(244226 = 100%) 2.115min, Scan: 120, 100: 1000, Ion:3225 us, RIC:8.066e +6. And in spectrum 1B with maximum Base peak: 274.9 (1.456e+6=100%) 2.799 min, Scan: 162, 100: 1000, ion: 1123 us, RIC: 1.882e+7.

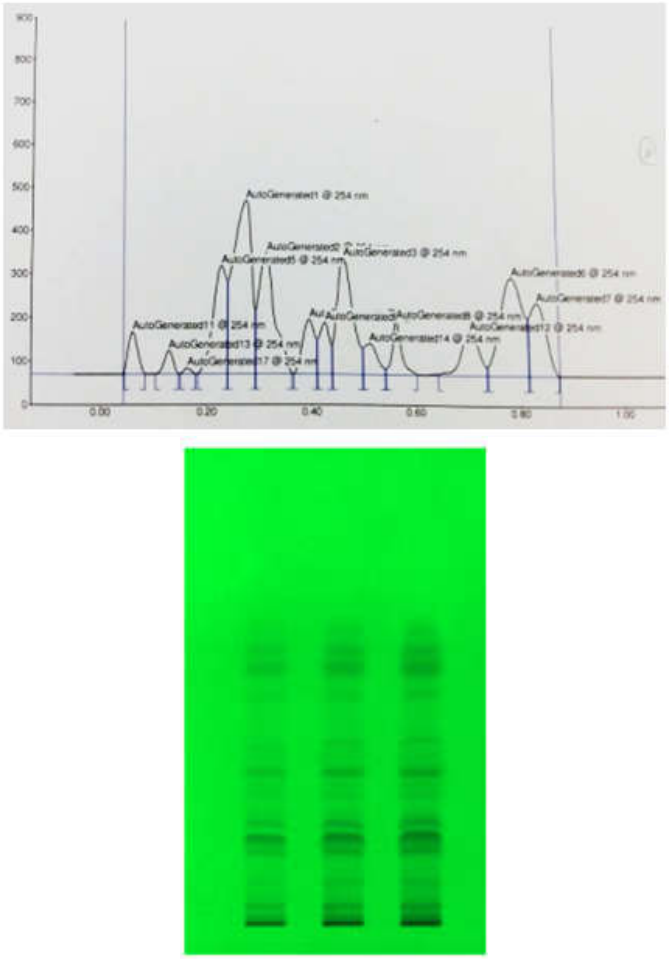

Fig. 17. Graphical representation of HPTLC result of Kimia Ethyl acetate fraction spectra and image of derivatized TLC plate at 254nm

The LC-MS spectra shows 28 peaks and from spectrum 1A with maximum Base peak 513.6 (2.971e +6= 100%) 12.470min, Scan: 760, 100: 1000, Ion:674 us, RIC:2.827e +7. And in spectrum 1B with maximum Base peak: 513.7 (2.545e+6=100%) 13.627 min, Scan: 832, 100: 1000, ion: 909 us, RIC: 2.325e+7. The LC-MS spectra shows 24 peaks and from spectrum 1A with maximum Base peak 303.1(1.345e+6 = 100%) 9.152 min, Scan: 120, 100: 1000, Ion: 1585 us, RIC:1.358e +7. And in spectrum 1B with maximum Base peak: 305.0(3.639e+6=100%) 10.362 min, Scan: 629, 100: 1000, ion:940 us, RIC: 1.816e+7 (Yun Jeong Hong, Tomas-Barberan, 2006)

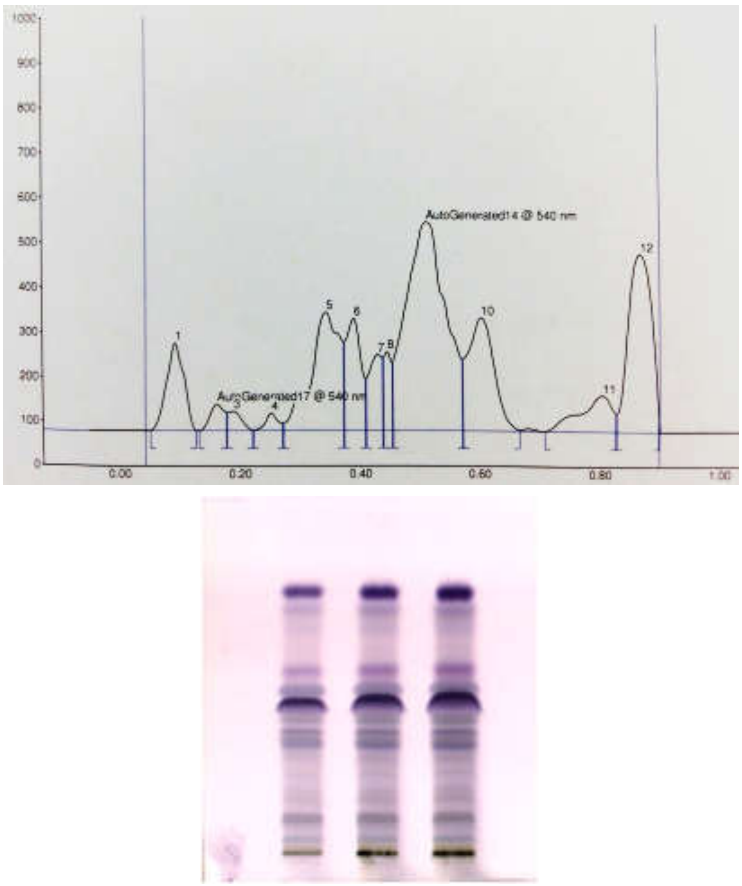

Fig. 18. Graphical representation of HPTLC result of Kimia Ethyl acetate fraction spectra and image of derivatized TLC plate at 540nm

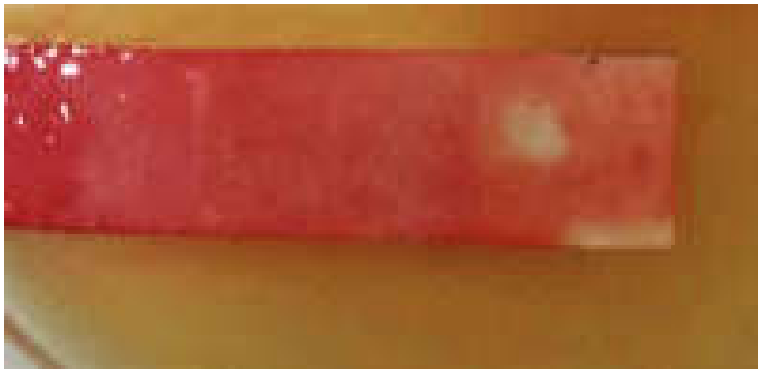

Fig. 19. Bioautography result of Fruit I (Kimia dates) Ethyl acetate fraction

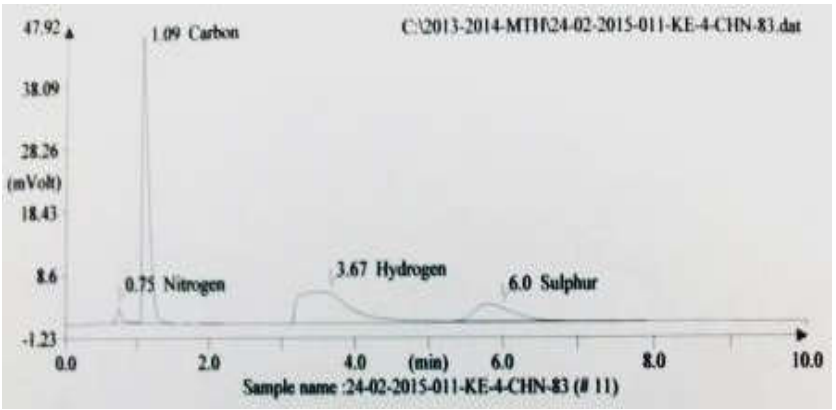

Fig. 20. CHNS spectra of Kimia Ethyl acetate fractiion

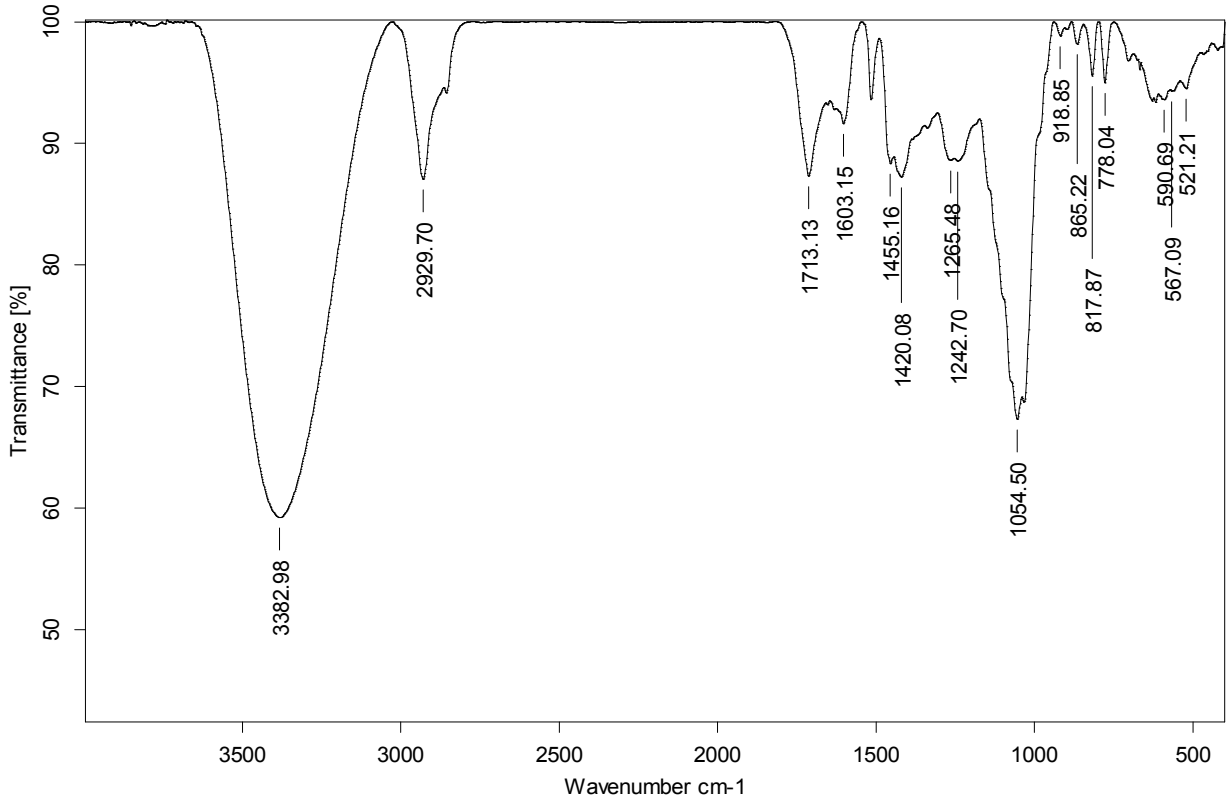

Fig. 21. FTIR spectra of Kimia Ethyl acetate Fraction

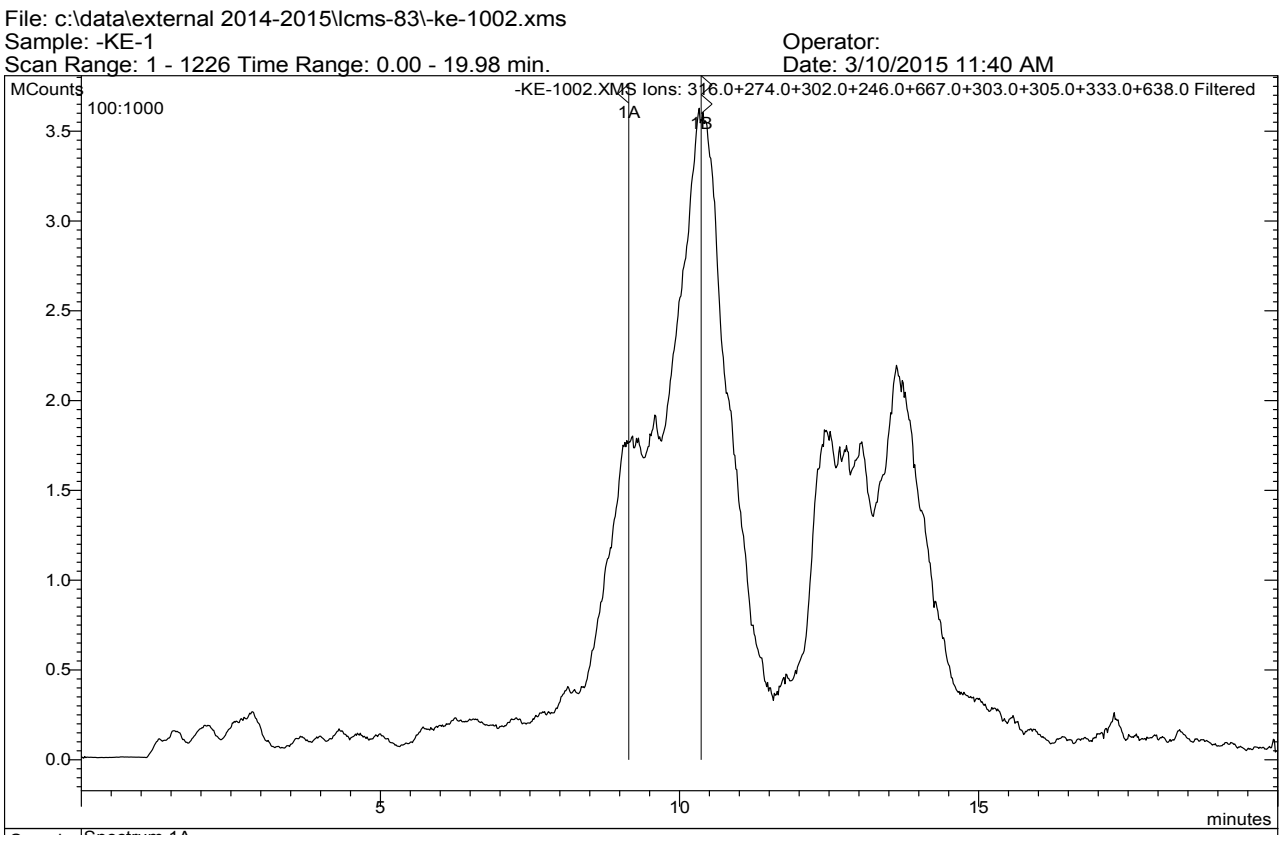

Fig. 22. LCMS Spectra of Kimia dates ethyl acetate fraction

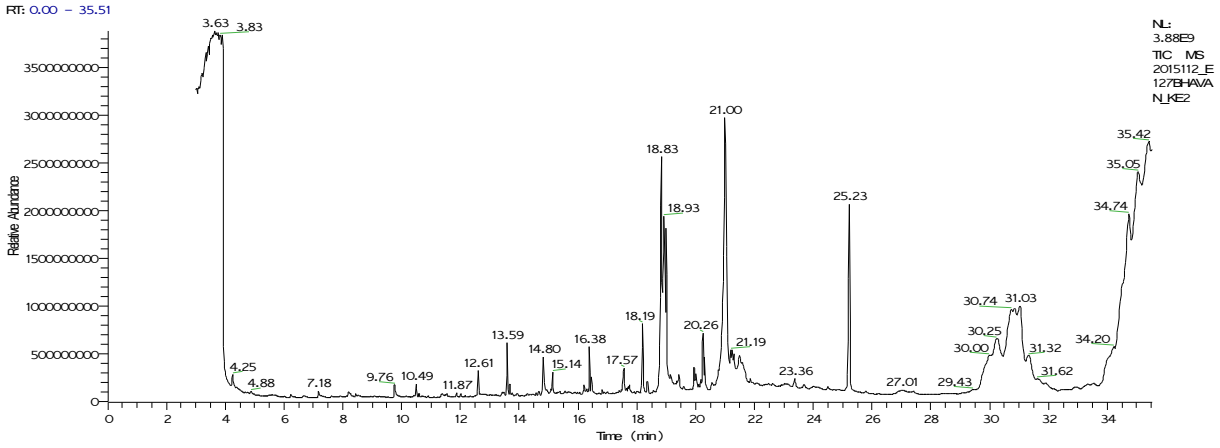

Fig. 23. GC-MS Spectra of Fruit I (Kimia dates) Ethyl acetate fraction

Table 1. Result of HPTLC and Bioautography of Kimia dates Ethyl acetate Fraction at 254 nm, 366nm and 540nm

Peaks @ 254nm	Rf@ 254 nm	Area	Area% 254nm	Peaks @ 366nm	Rf @ 366 nm	Area	Area% 366nm	Peak @ 540	Rf @ 540	Area	Area% 540nm	Bioautography result of coinciding Rf values
1	0.05	1294.5	2.53					1	0.05	4046.7	5.81	
2	0.10	645.4	1.31					2	0.13	997.9	1.43	
4	0.18	4639.3	9.39					3	0.18	681.3	0.98	
5	0.24	10245.9	20.74					4	0.22	569.4	0.82	
6	0.29	6338.0	12.83					5	0.27	9236.5	13.26	
7	0.37	2171.7	4.40					6	0.37	4582.3	6.58	
8	0.41	1738.9	3.52					7	0.41	2745.6	3.94	
9	0.44	6019.5	12.18					8	0.44	1660.2	2.38	
10	0.50	1304.7	2.64					9	0.46	22863.7	32.83	
11	0.55	1561.2	3.16					10	0.57	7906.6	11.35	
12	0.65	2461.5	4.98					11	0.71	3169.2	4.55	0.75
13	0.74	7187.9	14.55					12	0.83	11184.4	16.06	
14	0.82	3711.8	7.51	1	0.80	840.7	100					

Table 2. Result of CHNS analysis of Kimia Ethyl acetate fraction

Peak No.	Retention Time (min)	Area (0.1*uV*sec)	Element %	Component
1	0.750	127512	2.060	Nitrogen
2	1.092	2673294	23.711	Carbon
3	3.667	2713098	8.432	Hydrogen
4	6.000	908144	20.884	sulphur

Table 3. FTIR Spectra Of Kimia Ethyl acetate Fraction showing Functional Group

Major Peaks No.	Peak Range	Functional Groups
1	3382.98	N-H-1^0,2^0, amines, amides
2	2929.70	C-H stretching- alkanes
3	1713.13	C=O stretching - carbonyls and carboxylic acids
4	1054.50	C-N stretch -aliphatic amines

Table 4. Peak values of LC-MS Spectra of Kimia Ethyl acetate fraction

Peak no:		Spectrum 1 A	Peak no:		Spectrum 1 B
1.	Base Peak : 218.7 (244226= 100%) 2.115 min, Scan:120, 100: 1000, Ions: 3225 us, RIC: 8.066e+6	108.8	1	Base Peak : 274.9 (1.456 e+6= 100%) 2.799 min, Scan:162, 100: 1000, Ion: 1123 us, RIC: 1.882e+7	102.0
2.		136.1	2		181.4
3.		218.7	3		274.9
4.		225.7			
5.		316.7	4		290.9
6.		318.0	5		309.0
7		328.9	6		368.0
8		444.4	7		441.4
9		509.2			
10		580.6	8		532.3
11		622.4	9		614.1
12		689.9	10		689.3
13		697.9	11		776.1
14		764.0			
15		830.0	12		850.9
16		949.6	13		927.6

Table 5. Result of GCMS of Fruit I (Kimia dates) Ethyl acetate fraction

Peak No.	Retention Time	Name of Compound	Chemical Formula	Molecular Weight	Probability	Activity
1	18.83	1,2- Benzenedicarboxylic acid, butyl 8-methylnonyl ester	$C_{22}H_{34}O_4$	362		Antibacterial, antifungal, antiviral, antioxidant, fungi toxic, cytotoxic and antifouling activity and anti-inflammatory
2	21	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}0_2$	308		Antioxidant, hepatoprotective, antihistaminic, hypocholesterolemic, antieczemic, insecticidal and anti-feedant, antiarthritic, antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne
3	25.23	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}0_4$	390		antimicrobial, antifouling
4	30.25	12-Oleanen-3-yl acetate, (3α)	$C_{32}H_{52}O_2$	468	73	
5	31.03	12-Oleanen-3-yl acetate, (3α)	$C_{32}H_{52}O_2$	468	75	Antimicrobial, anti-diabetic, anti-amylase inhibitor, antioxidant, antibacterial, anti-inflammatory, antitumor activities
6	34.74	12-Oleanen-3-yl acetate, (3α)	$C_{32}H_{52}O_2$	468	66	
7	35.42	12-Oleanen-3-yl acetate, (3α)	$C_{32}H_{52}O_2$	468	69	

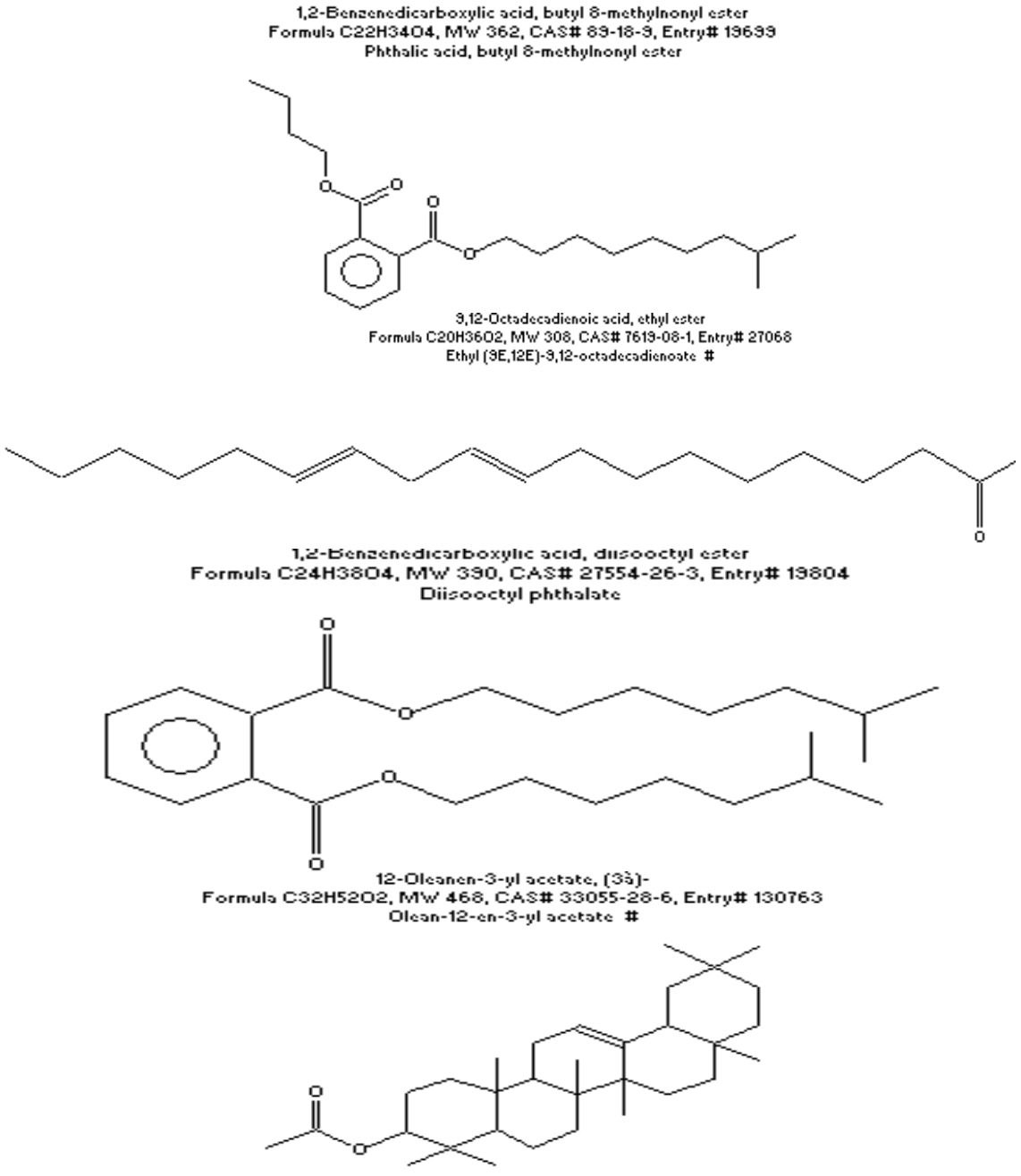

Figure 24. The structure of main compounds identified by GC-MS Fruit I (Kimia dates) Ethyl acetate fraction

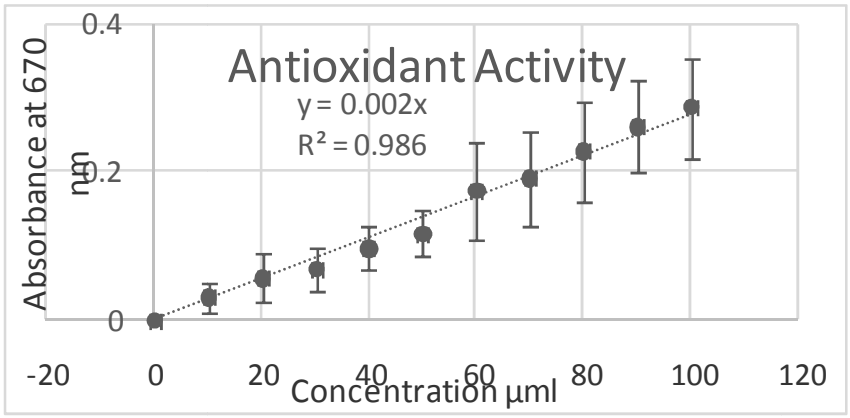

Figure 25. Graphical representation of antioxidant activity of Kimia dates Ethyl acetate fraction

Fig. 26. MIC of Antiviral activity of Kimia dates Ethyl acetate fraction

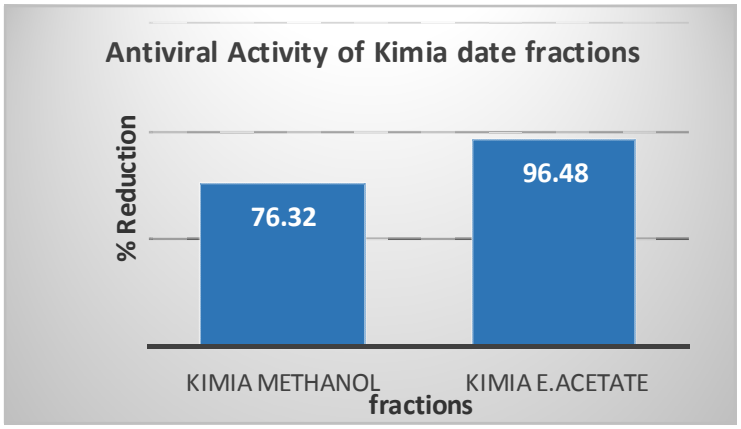

Fig. 27. Antiviral activity of Kimia dates Ethyl acetate fraction

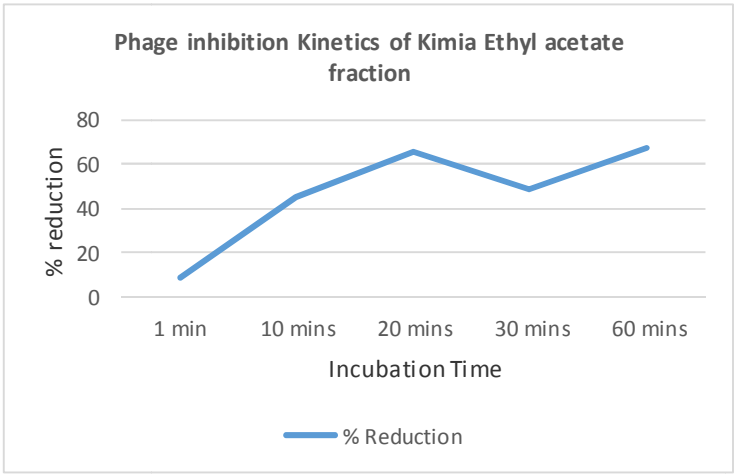

Fig. 28. Phage inhibition kinetics of Kimia ethyl acetate fraction

The Result from **GC-MS** spectra of Kimia dates Ethyl acetate fraction showed 34 peaks indicating the presence of thirty four compounds, out of which 7 major compounds described and identified at Rt.35.42 minutes reveals the presence of 1, 2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester, 9, 12, 15-Octadecatrienoic acid, ethyl ester 1, 2-Benzenedicarboxylic acid, di isooctyl ester (probability-36%), 12-Oleanen-3-yl acetate, (3α) (probability-75%). (3, 7, 18, 19, 21, 36, 40) On the basis of HPTLC, CHNS, FTIR, LC-MS, GC-MS analysis, inference may be drawn that 12-Oleanen-3-yl acetate, (3α) ($C_{32}H_{52}0_2$) with probability-75% may be the bioactive compound in the ethyl acetate fraction, belonging to the class triterpenes, showing a wide range of antimicrobial, anti-diabetic, anti-amylase inhibitor, antioxidant, antibacterial, anti-inflammatory, antitumor activities.

Antioxidant levels of in the present study reported that *Phoenix dactylifera* might have the ability to supress the free radicals. The Kimia dates, Fruit I ethyl acetate fraction (D) showed the maximum antioxidant activity (98.57µg/ml) Vennila, 2014)

The ethyl acetate fractions of fruit I showed promising antiviral activity with MIC 6.25 mg/ml (Kimia dates). Then the nature of inhibition was evaluated by performing kinetics of the Kimia fraction on the phage infectivity. (Tambe Rashmi *et al*., 2013) In the earlier studies, *Phoenix dactylifera* L date demonstrated antiviral activity with an MIC value of <10 µg ml^{-1} for the *Pseudomonas* phage ATCC 14209-B1. (Yun Jeong Hong and Tomas-Barberan, 2006) The Fruit ethyl acetate fraction show a strong ability to inhibit and reduce 96.48% infectivity of *coli* phage and completely prevented bacterial lysis at 30 minutes which it is hoped will promote research into its potential as a novel antiviral agent against pathogenic human viruses. (Yun Jeong Hong and Tomas-Barberan, 2006)

Based on the maximum inactivation of *phage* by Fruit I, ethyl acetate fraction leading to the evaluation of phage inhibition kinetics. The effect of extract on phage's life cycle shows 8.78% inhibition after 1 minute exposure, while approximately 65.6% at 20 min of exposure. This is the probability that the one life cycle of *coli phage* completes approximately 20 minutes indicating the effect of fraction may be on release of from the host. The Kimia ethyl acetate fraction then subjected to find its antibacterial activity against MDR human pathogens but the present study did not exhibit antibacterial activity against all MDR pathogens (Dubey and Padhy 2013, and Sahu *et al*., 2015).

Conclusion

In recent years , an explosion of interest in the numerous health benefits of dates has led to many studies , identification and quantification of various classes of phytochemicals with a great potential uses in the booming industries of functional foods and nutraceuticals. Researchers have found that phytochemicals have the potential to stimulate the immune sustem, prevent toxic substances in the diet from becoming carcinogenic, resuce inflammation, prevent DNA damage and aid DNA repair, reduce oxidative damage to cells, slow the growth rate of cancer cells, trigger damaged cells to self- destruct (apoptosis) before they can reproduce, help regulate intracellular signiling of hormones and gene expression, and activate insulin receptors. The study has revealed the presence of phytochemical constituent like Tannins, Saponins, Flavonoids, Glycosides, Alkaloids and Terpenoids. Out of these presence of high levels of compounds from Terpenoids family indicates Antimicrobial, antiviral and antioxidant potential. The bark fraction also display high levels of antibacterial activity against MDR pathogens. The presence of Vitamin E which is a fat-soluble strong antioxidant was also detected. It is expected that the antiviral property of bark methanol fraction will be of great significance in further refinement of antiviral drug design and development as potential bio therapeutic agents against medically important pathogenic human viruses, such as the human immune deficiency virus (HIV). As such the findings elucidated in the present study are expected to find practical application in diverse fields such as pharmaceuticals, food processing, nutraceuticals, Ayurveda, cosmetics, biotechnology, fisheries, nanomedicine, agriculture, bio pesticide, green chemistry, phytomedicinal research etc. We fervently hope that this study will contribute in a small but significant way to the ever expanding realm of knowledge and research in the field of Microbiology and Phytomedicinal research.

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