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Phytochemistry, antioxidant and antibacterial activities of fruit extracts of *Myrsine africana* L.

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Abstract: *Myrsine africana* L. a commonly consumed medicinal plant grows in forest of mountains region located at North East of Pakistan. In current study, the fruit extracts were chemically characterized and their bioactivities were determined. Higher quantity of total phenols, total flavonoids and tannins were obtained from methanolic fruit extracts. The HPLC analysis provided higher level of quercetin followed by rutin and p-coumaric acid. Whereas the GC-MS quantification had given significant level of ten saturated and unsaturated fatty acids and some of them were not reported earlier. *In vitro* study, lower cytotoxic behavior of fruit extracts but higher antioxidant values as well as higher zone of inhibition versus *S. aureus*, *E. coli*, *K. pneumonia* and *B. subtilis* and *Mycobacterium tuberculosis* were observed. The organic compounds found in fruit extracts of *M. africana* correlated well with its used in ethno medicines.

Keywords: *Myrsine africana,* chromatography, extraction, bioactivities.

INTRODUCTION

Myrsine africana L. (Myrsinaceae), locally recognized *as Khokhal.* It is widely distributed in Asia and Africa, a plant of the drier tropics and subs tropic (Lall *et al.,* 2017; Gul *et al.,* 2017). It is found at elevation upto 3,800 meter and can tolerate temperature 22-35ºC and preferably annual rain fall in the range of 600-800 mm. It is common in summer and winter rainfall areas of North Eastern Part of Pakistan. It is naturally growing on rocky sides in forests and evergreen shrub up to 2 meters high. The fruits are eatable, and are conventionally utilized as a carminative, taster, spicing mediator and cologne. The fruit and leaves extracts are used against many human aliments like blood decontaminant, toothache, bronchitis, cough, tuberculosis as well as many others microbial diseases. The antiseptic effects of leaf and fruit of *M. africana* have been described earlier (Ahmad *et al*., 2016) and isolation of flavonoids and flavonoid glycosides studied by Kishore *et al.,* (2018). The myrsigenin has been extracted from methanol extract of *M. africana*, those have been shown to be antispasmodic (Azam *et al*., 2011). The reports of World Health Organization revealed that 80% of people round the world depended on therapeutic agents obtained from plant sources and many existing drugs in markets were prepared from phytochemicals (Ekor, 2014). These therapeutic drugs include digitalis, ergotamine, quinine and salicylates etc.

Pakistan is one of countries where production of several medicines based on various plant species become now rarer due to over exploitation (Yousaf *et al.,* 2018; Mustafa *et al.,* 2019; Khadam *et al.,* 2019). Therefore conservation of these plants having endanger condition of existence must be cultivated to maintain their production.

To our knowledge chemical composition and bioactivities of fruit of *M. africana* is uncommon in literature. Therefore present study was managed to examine the fruit extract *M. africana* with HPLC and GC-MS, to explore antioxidant, cytotoxic, antibacterial and anti-tuberculosis, activities of fruit extracts of this plant.

MATERIALS AND METHODS

Plant material

The fruit samples of *Myrsine africana* were collected from Kotli Sattian (North Eastern Punjab), a mountain region of Pakistan. This area is considered as arid zone and annual rain is comparatively better than other parts of the country. Plant samples were identified by expert taxonomist from Department of Botany and voucher specimen (No.131) was deposited to department. The plant materials were shadow and sun dried followed by oven drying and grounded to a powder. A total 100g of sample was macerated with distilled water, methanol, ethanol, chloroform and n-hexane using method of Soxhlet apparatus and a rotary evaporator (BUCHI Rotavapor® R-300).

Phytochemical analysis

Total flavonoids, phenols and tannins were estimated using a modified Folin-Ciocalteu colorimetric methods as described earlier by Abbasi *et al.* (2015).

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HPLC analysis

The HPLC investigation was carried out with Shimadzu HPLC system (Tokyo, Japan) equipped with C18 column (25mm × 4.5mm, 5µm) and UV/visible detector. The components were eluted using a gradient of acetonitrile and 0.1% phosphoric acid (36:64). The injection volume for all samples was 20µl. The flavonoids were checked at 280 nm and 285nm at a flow rate of 1 ml/min. The quercetin was used as a standard and all determinations were performed in triplicate.

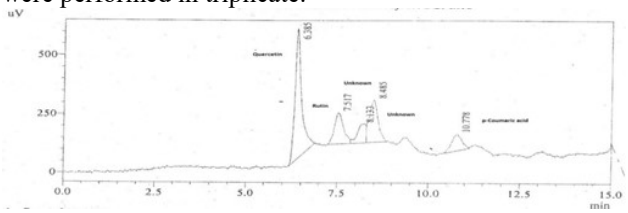

Fig. 1: The HPLC profile of flavonoids from methnolic fruit extract of *M. Africana* quercetin was used as standard

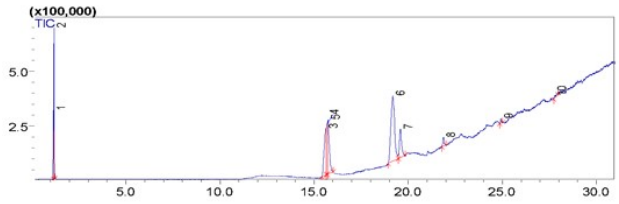

Fig. 2: GC-MS profile of constituents of methanolic Fruit extract of *M. Africana*

GC-MS analysis

The details of GC-MS (Shimadzu) analysis were: capillary column RTx- 5MS, 30m x 0.25mm x 0.25µm, split injection at 250ºC, helium carrier gas, column flow 1.2mL/min at a constant linear velocity mode. The program of column oven temperature was preset at 4ºC/ min to 150ºC. The carrier gas used was N_2 (1.0mL/min), 0.2µl injection volume and splitting share fraction was 50:1 at 275ºC. The fruit extract was derivatized and identified compounds were compared with spectral data base of "The National Institute of Standards and Technology" (NIST). Each compound (%) was obtained after the comparison of peak area with the total peak areas.

Antioxidants activity

The measurement of 2,2- diphenyl-1- picrylhydrazyl (DPPH) activity was obtained by the procedure reported by Brand-Williams *et al*., 1995. The sample was reacted with the stable DPPH compound in an ethanol solution and percentage (scavenging activity) was determined by the method proposed by (Mensor *et al*., 2001). The 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay was done by following the procedure of (Ashafa *et al.,* 2010). The superoxide radical, hydroxyl radical and Fe chelating assays were applied according to (Cefarelli *et al.,* 2006; Dehghan and Khoshkam 2012). On providing 50 % inhibition, (IC_{50} µg/ml) was calculated from dose response curve.

Brine shrimps assay

The cytotoxicity of samples was tested according to method reported by Ruch *et al*., (1989) and DMSO was used as control

Antibacterial activity

The fruit extracts of M. *Africana* were partitioned to examine antibacterial potential through agar well diffusion method against four bacterial strains such as *Staphylococcus auresu* (ATCC 6538), *Klebsiella pneumonia* (MTCC618), *Escherichia Coli* (ATCC15224) and *Bacillus subtilis* (ATCC6633). These bacterial strains were chosen on the basis of their clinical and pharmacological importance as reported by (McCracken & Cowsan, 1983). The standard antibiotic drug (Cefixime) was used and OD was taken at 420 nm along with UV/*Visible* spectrophotometer. The lowest concentration of the extracts was estimated as MIC that hindered the growth of bacteria in 24 hours of incubation period (Upadhyay, 2015).

Anti-Mycobacterium tuberculosis assay

The drugs resilient strains of *Mycobacterium tuberculosis*, bg 206 and bg 1972 along with a sensitive strain H37Rv were used in this experiment. The inoculums of all strains were prepared. From the dilution (10^{-2}) of each strains of *Mycobacterium tuberculosis* suspensions, about 60µl was streaked on the Lowenstein Jensen (LJ) medium slants using loop with 3mm external diameter. The fruit extracts (05mg/ml, 10mg/mL and 50mg/mL) were separately incorporated in the medium. The anti-mycobacterial activities of fruit extracts were carried out by using reported method (Martins *et al.,* 2014).

STATISTICAL ANALYSIS

The data obtained during various analyses were analyze statistically for various parameters.

RESULTS

The various fruit extracts were subjected to preliminary phytochemicals screening and according to results obtained methanolic fruit extracts of *M. africana* comprised of greater quantities of flavonoids, total phenols and tannins as compared to other extracts analyzed (table 1). The phytochemicals are naturally occurring chemical compounds produced by plants and acquired health benefits for consumers (Baskaran *et al*., 2011). Many phytochemicals protects human body from inflammation, oxidative stress and several other diseases including cancer if fruits and vegetables are consumed regularly (Abbasi *et al*., 2015; Ujowundu *et al*., 2008).

Table 1: Phytochemicals from fruit extracts of *M. africana*

Extract	Total flavonoids (mgGA/100g)	Total phenols (mg GA/100g)	Tannins (mg GA/100g)
Methanol	18.16 ± 2.13Þ	22.31 ± 1.23[a]	5.13±0.33Þ
Ethanol	15.13± 1.32[a]	13.71 ± 1.34[a]	5.85±1.72[a]
Chloroform	12.35 ± 0.24Þ	14.06 ± 1.5Þ	4.58±0.71[a]
Aqueous	4.13 ±0.56	2.18± 0.58	0.91±0.43

Mean ± SD (n=3), [a] = p<0.01,Þ= p<0.05.

Table 2: GC-MS analysis of Organic compounds from methanolic fruit extracts of *M. africana*

Peak	R.T	Area (%)	Molecular Formula	Molecular weight	Name of compound	NIST Lab no.
1	0.321	6.07	$C_{27}H_{56}$	396	Heptacosanol	1l Lib
2	1.167	25.95	C_2H_4O	46	Ethanol	11 s Lib
3	1.247	1.73	$C_4H_{10}O$	74	Isobutyl alcohol	11 Lib
4	15.889	24.26	$C_{38}H_{68}O_8$	652	Ascorbic acid	11 Lib
5	19.352	24.36	$C_{18}H_{34}O_2$	282	Octadecenoic acid	11s Lib
6	19.760	5.59	$C_{18}H_{36}O_2$	284	Stearic acid	11s Lib
7	22.025	3.02	$C_{16}H_{31}O_{10}$	274	Palmitoyl chloride	11s Lib
8	22.595	3.28	$C_{19}H_{32}O_2$	842	Hexacontane	11 Lib
9	25.087	2.14	$C_{44}H_{84}O_8$	785	Phosphaheptacos	11 Lib
10	26.106	3.60	$C_{31}H_{55}F_7O_2$	592	Hepatocosyheptafluoro Butyrate	11Lib

Table 3: Antioxidant effects of fruit extracts of *M. africana* (IC50 values µg/ml)

Extract 100 µg/ml	DPPH	H_2O_2	ABTS	Reducing power	Superoxide	Iron chelation
Ethanol	39.17±0.26[a]	44.51±0.18a[a]	45.72±3.71[a]	67.53± 2.16[a]	108.54± 1.65 Þ	34.32± 1.35[a]
Methanol	18.23±0.45Þ	41.25±2.15[a]	39.42±2.38[a]	61.18± 3.55 Þ	101.23± 1.05 Þ	22.64± 2.35 Þ
Chloroform	26.35±0.48 Þ	43.62±2.81[a]	41.21±1.75[a]	72.44± 2.65 Þ	105.04± 1.25 Þ	26.08± 3.16 Þ
Aqueous	42.15±0.36[a]	52.43±3.45 Þ	48.51±3.38[a]	92.16± 3.64[a]	114.18± 1.03[a]	41.35± 1.26[a]
Ascorbic acid	9.26±0.71[a]	7.52 ±0.39[a]	6.34±0.35 Þ	11.35 ±1.17 Þ	33.25± 2.48[a]	35.06± 1.28[a]
Gallic acid	4.91±0.36 Þ	1.68±0.23 Þ	1.23±0.34 Þ	2.35±0.38 Þ	18.26±1.66 Þ	20.38±1.38 Þ

Means ± SD, (n = 3), whereas [a] = p<0.01,Þ= p<0.05. Gallic acid was used as standard

Table 4: Concentration (µg/ml) of fruit extracts used for cytotoxicity

Fruit Extracts	10	100	1000	LD50
Ethanol	38.2±0.5	48.3±0.4	55.3±0.7	<1000
Methanol	36.5±0.4	43.1±0.5	51.7±0.5	<1000
n-hexane	45.7 ±0.6	47.8±0.4	58.7±0.5	700
Chloroform	49.6±0.5	47.6±0.5	48.3±2.1	100
Aqueous	48.5±0.6	52.5±0.76	58.9±0.5	270
DMSO	0.0	0.0	0.0	0.0

Values are Mean ±SD, (n=3), DMSO was used as standard

Table 5: Antibacterial activities of various fruit extracts; zone of inhibition in mm

Extracts	*S. aureus*	*E. coli*	*K. pneumonia*	*B. subtilis*	Roxithromycin	Cefixime
Ethanol	19.6±0.6	18.4±0.9	19.5±0.3	22.6±0.4	16.5±0.9	12.5±0.3
Methanol	23 .5±0.7	22.5±0.97	22.9±0.5	21.7±0.3	13.6±0.9	11.5±0.3
n-hexane	18.3 ±0.6	18.7±0.5	18.8±0.4	17.5±0.8	13.8±0.5	10.8±0.5
Chloroform	19.8±0.5	18.7±0.6	15.4±0.6	16.6±0.7	14.5±0.5	11.8±0.7
Aqueous	11.5±0.6	13.5±0.68	12.7±0.8	12.5±0.6	11.6±0.5	10.6±0.7

Results mean± S D, (n=3).

Table 6: MIC values minimum inhibitory concentration (µg/ml) for various bacterial strains against fruit extracts

Extracts	*S. aureus*	*E. coli*	*K. pneumonia*	*B. subtilis*	Roxithromycin	Cefixime
Ethanol	0.7±0.3	1.5±0.4	0.8±0.3	1.6±0.5	1.3±0.7	1.5±0.5
Methanol	0.3±0.1	2.3±0.4	0.7±0.3	1.3±0.4	1.4±0.5	1.1±0.7
n-hexane	1.5 ±0.3	1.6±0.8	0.9±0.5	1.4±0.8	1.5 ±0.4	1.3±0.6
Chloroform	1.8±0.6	1.7±0.5	1.4.±0.5	1.5±0.7	1.5±0.3	1.3±0.8
Aqueous	1.2±0.6	1.6±0.7	1.6±0.4	1.8±0.5	1.5±0.4	1.5±0.5

Results are Means ± SD, (n = 3)

Table 7: Anti-tuberculosis activities of fruit extracts on LJ Media

Extracts	Isolates	Mean CFU on media				Percentage Inhibition		
Methanolic fruit extracts	H37Rv	140	80	76	20	43	46	86
	bg 206	150	90	60	35	40	60	77
	bg 1972	130	86	64	40	34	51	69

Cc / Ct = No of colony / No of colony in the control media slope inhibition (Cc-Ct/Ccx 100).

HPLC analysis

The HPLC analysis revealed that methanolic fruit extracts contained quercetin rutin and *p*-coumaric acid (fig. 1). The higher level of flavonoids in fruit extract exposed the usefulness of *M. Africana* fruit in local medicines. Similar results were also reported by Movileanu *et al.* (2000) after analysis of other plant extracts.

GC MS analysis

Methanolic fruit extracts *of M. Africana* were subjected to GC-MS analysis and results are presented in fig. 2 and table 2.

Ten organic compounds were detected by GC-MS analysis (fig. 2) and among that 7 compounds were dominating in fruit extract (figs. 2 and table 2). By comparing chromatogram of GC-MS, the peak areas of compounds were calculated and other required information were obtained from NIST library data base. The peak 1 Indicates Heptacosanol (6.07 %) $C_{27}H_{56}$, Peak 2. Ethanol (25.95%), C_2H_4O, peak 3 Isobutylalcohol (1.73%) $C_4H_{10}O$, peak 4 Ascorbic acid (24.26%), $C_{38}H_{68}O_8$, peak 5. Octadecenoic acid (24.36%), $C_{18}H_{34}O_2$, peak 6 Stearic acid (5.59%), $C_{18}H_{36}O_2$, peak 7 Palmitoylchloride (3.02%), $C_{16}H_{31}$ O_{10}, peak 8 Hexacontane (3.28%), $C_{19}H_{32}O_2$, peak 9 Phosphaheptacos (2.14%). $C_{44}H_{84}O_8$, and peak 10 Heptacosylhepta fluorobutyrate (3.60%) C_{31} $H_{55}F_7O_2$. The compounds on peaks 8, 9 and 10 were not reported from plant extracts especially from *M. africana* or its relevant plant species, therefore, analysis of these compounds is first investigation from fruit extracts of *M. africana* and this claim is on the basis of extensively survive of available literature, similar results were also reported by other authors from other plant extracts (Mani & Murugan 2017). Organic compounds along with Retention time (R.T) and Peak area (%) obtained by GC-MS analysis are included in the table 2.

Antioxidant assays

The antioxidant activities of fruit extracts of *M. Africana* were evaluated. The free radical scavenging activity of methanol fruit extract in DPPH assay appeared lower (IC50 18.23±0.45µg/ml) than in the other assays indicating higher antioxidant activities of methanol formulating of this fruit (table 3).

Cytotoxicity assay

For the assessment of brine shrimps cytotoxicity assay, various concentration of fruit extracts (10,100 and 1000 µg/ml) were prepared (table 4). The results indicated brine shrimps larvicidal potential and it was observed that lethality was maximum at higher concentration of fruit extracts and directly depends on concentration of extracts. It is expected that fruit extracts probably comprised of an antitumor chemical composites in the form of important nutrients. The fruit formulation having values LD_{50} <1000 µg/ml was considered as effective while LD_{50} >1000 µg/ml was biologically non-toxic. According to results, higher mortality was noted for n-hexane and chloroform fruit extracts and these results were comparable with results reported previously (Sandeep *et al.,* 2012).

Antibacterial activities of fruit extracts

The activity of methanolic fruit extracts to inhibit growth of bacteria strains like *S. aureus*, *E. coli*, *K. pneumonia* and *B. subtilis* strains was assessed and compared with standard antibiotics (table 5). All fruit extracts had shown noteworthy antibacterial capability for Grams +ve and Gram -ve bacteria strains, suggesting that *M. africana* fruit extracts comprised of antimicrobial natural compounds like flavonoids *etc.,* that can be used to overcome infectious diseases in human population. Therefore, present investigation supports the pervious information reported by other authors with slight variations (Fawole *et al*., 2008).

The resistance of various bacterial strains is a main risk to health of general population. The medicinal plants are being anciently consumed by human population to get remedies from communicable sickness by indigenous cultures worldwide and have the ability to deliver applicable healing for antibiotic-resistant illness (Shah *et al.,* 2019; Muniandy *et al.,* 2019; Baskaran *et al*., 2011).

The lower level of inhibitory concentration specifies extensive capacity of fruit extracts to inhibit microbial growth. The MIC values of various extracts exposed variability in the inhibitory concentrations of each fruit solution. The lower level of MIC obtained for methanolic fruit extracts was possibly owing to purity of fruit materials in these solvents (table 6). Therefore findings of antimicrobial capacity of fruit extracts obtained in this study were comparable to results reported previously by other authors (Guessan *et al.* 2007).

Anti-Mycobacterium tuberculosis assays

Three different concentrations of fruit extracts were used to determine their activities against three strains of *Mycobacterium tuberculosis* like H3Rv, bg 206 and bg1972 (table 6). After eight weeks of incubation, each slope was finally observed for number of colony forming unit (CFU) (McCracken & Cowsan, 1983). The colonies of each strain on media containing extracts were compared with the extract free slope of that specific strain and the percentage inhibition was calculated. The pronounced anti-tuberculosis activity of *M. africana* fruit extracts was observed which indicated the usefulness of this fruit against various chest relevant diseases.

DISCUSSION

The biologically active compounds available in the plant extracts are being used as foods and medicines. The interest is growing for isolation, depiction and the *in- vivo* and *in -vitro* assessment of biological activities of these phytochemicals, those either alone and /or in combination, have tremendous therapeutic potential for curing of various human aliments. Consequently varieties of chemical compounds attained by analysis of fruit extracts of *M. africana* impart significant anti proliferative, cytotoxicity, antioxidants, antibacterial and anti-tuberculosis activities. The HPLC analysis indicates that the major compounds found in fruit extracts of *M. africana* were quercetin, rutin and p-coumaric acid. Which together with fatty acids and other organic compounds obtained by GC-MS are likely to be the main antioxidants. The flavonoids acquired many biochemical properties and can prevent the damaging processes caused by oxidative stress (Cefarelli *et al.,* 2006). The Brine shrimp lethality bioassay indicates non toxicity of fruit extracts and its suitability use in folk medicines (Ujowundu *et al.,* 2008). The antibacterial and anti-mycobacterium study indicates that fruit extracts of *M. Africana* exhibited healthier activities at elevated concentration against tested strains. The judgment of zone of inhibition was higher than results reported by other authors (Baskaran *et al.,* 2011). The previous studies specifies association with reduction in cancer risk with consumption of fruit and vegetables rich in flavonoids as earlier reported by (Park *et al.,* 2008; Ferguson *et al.,* 2004). Therefore, flavonoids act as antioxidants, antibacterial, antiviral and anti-inflammatory activates. The flavonoids provide antioxidant defense mechanism and stimulate protective enzymes system in body. In this way flavonoids act as safe guard against disease such as cancer, cardiovascular, respiratory tract, arthritis and aging factors. Normally flavonoids have polyphenolic structure that makes it suitable for different pharmacological activities. The hydroxyl functional group of flavonoids shows antioxidant activates by scavenging free radicals or chelating melting ions and help to stop realizing free radical leading to damage cells those more causes serious diseases in human body.

CONCLUSION

The chemical composition and biological activities of fruit extracts *M. africana* are reported for the first time. The results reflected that fruit contained useful organic compounds having health benefits. Because of frequent utilization in ethno medicines, this useful shrub should need conservation.

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