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Evaluation of total flavonoid and phenolic amount, antioxidant, antimicrobial and cytotoxic potential of *safoof-e-tabkheer*

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***Abstract*---**We determined the different parameters of antioxidant activity with different solvents of polarities in sequence of raising polarity i.e., pet-ether, chloroform, ethyl acetate, methanolic and aqueous extracts of *Safoof-e-Tabkheer*. Potential antioxidant was detected through DPPH, nitric oxide and metal chelating test. *MeSt* and *AqSt* extract had important antioxidant activity in compared to all extracts, by values of IC_{50}=82.31±2.16µg/mL, IC_{50}=98.65±1.47µg/mLin the DPPH and IC_{50}=84.28±1.54µg/mL, IC_{50}=72.14±1.04µg/mL in nitric oxide assays, and IC_{50}=233.38±2.39µg/mL, IC_{50}=98.65±1.47µg/mLmetal chelating test. The total amount of phenol and flavonoid quantified were*MeSt*194.7±1.15mg/g and *AqSt* 149.4±1.64mg/g of gallic acid equivalent per gram respectively and *MeSt*152.4±1.12 mg/g and *AqSt* 105.01±1.13mg/gof rutin equivalent respectively. Antibacterial, antifungal and MIC were calculated. *MeSt* and *AqSt* prohibited the growth of Gram-negative and Gram-positive bacteria, at MIC of 8-64 µg/ml. Antifungal activity at MIC was 16-128 µg/ml. *MeSt* and *AqSt* exhibited cytotoxicity against MTT assay. These results suggest that *MeSt* and *AqSt* extract has potential cytotoxic, antimicrobial and antioxidant potential that support the ethnopharmacological uses of *Safoof-e-Tabkheer*.

Keywords*---Safoof-e-Tabkheer*, antioxidant activity, Antibacterial activity, Antifungal activity, Total phenolic and flavonoids, MTT assay.

1. Introduction

Antioxidant-prosperous dietary supplements or foods have the maximum possibility to decrease the cell damage through free radicals. A daily herbals antioxidants diet is very necessary to keep proper human health as poly herbals are a major source of antioxidant organic compounds.[1]Lots of herbal drugs commercially presented in the marketplace for the dealing within curable and curable diseases. By the use of antioxidant rich food diet can be decrease or avoid diseases i.e. aging. Large amounts of antioxidant compounds are discovered by herbal sources belong to diverse category of compounds including a huge range of chemical and physical properties must include in dietary supplements.[2]Grand awareness in recent timespaying attention on the adding up of flavonoids and polyphenols in biological and dietary systems, just because of scavenge free radicals capacity, i.e. power of antioxidant. The production of free radicals acting a significant task in the development of a grand amount of pathological trouble, like brain dysfunction, atherosclerosis, cancer and also has diverse effects on inflammatory diseases.[3] Since ancient time's terrestrial plants have been known potential sources antioxidants. Today compounds derived from plants source play a very important role for production of new chemical model introducing the clinical trials or market annually.[4] This is the time now that we introduce a novel and effective ingredient base poly herbal formulation for incurable diseases without any adverse effect.

No report on the antioxidant, cytotoxicity and antimicrobial activities of *Safoof-e-Tabkheer*(ST) is available at present. In this paper, I have reported that ST showed *in-vitro* antioxidant potential for example antioxidant activity, determination of total flavonoids (TFC), determination of total phenolics contents (TPC), radical-scavenging activity DPPH, Nitric oxide radical inhibition assay, metal chelating activity, antibacterial, antifungal and cytotoxic potential. Physicochemical standardization of *Safoof-e-Tabkheer* (ST) was done with modern tools.[5]

2. Materials And Methods

The marketed *Safoof-e-Tabkheer* Unani polyherbal powder wasobtained from Unani dawakhana, Srinagar, J&K.

2.1 Bacterial and fungal strains and culture media

Bacterial and fungal strains were obtained from Institute of Microbial Technology, Chandigarh, India (Microbial Type Culture Collection, MTCC). Antibacterial activity of all extracts of ST against *S. aureus* (MTCC-96), *P. aeruginosa* (MTCC-1688), *K. pneumonia* (MTCC-19), *S. typhi* (MTCC-98), *E. coli* (MTCC-739)and *P. vulgaris* (MTCC-426) were studied. Mueller Hinton Broth (Merck) and Mueller Hinton Agar was used for the grown of bacterial strains. The five fungal strains of *S. cerevisae* (MTCC-170), *A. fumigates (*MTCC 9001*), P. crysogenum (*MTCC 947*), H. viridescens (*SITCC-1*) and M. plumbeus* (SITCC-2)were grown in Potato dextrose agar 0.9% NaCl was used at 0.5 McFarland for cell suspension of microorganisms to obtain 10^6 cfu/ml approximately.

2.2 Antibiotics

Streptomycin (S 10µg/disc) and Fluconazole (FLC 25µg/disc) were used as standard antibiotics. Both antibiotics were obtained by Laboratory Hi-Media Private Ltd., Mumbai, India.

2.3 Chemicals

All the analytical grade chemicals were used was purchased by a local area dealer and some frommade by Hi-media private Ltd. Mumbai, India.

2.4 Extraction

100 gms of powdered ST was extracted by way of raising polarity order of solvents like pet-ether, chloroform, ethyl acetate, methanol and aqueous using apparatus soxhlet through hot successive extraction procedure.[6] Finally, extracts were concentrated using rota-evaporator and final residues dried in desiccator over Calcium carbonate. Practical yield was weighed and measured in triplicate.

2.5 Total phenols content

The amount of total phenolics compounds in ST extracts was detected from procedure Folin-Ciocalteu reagent with gallic acid as standard. In 0.5 ml of sample solution added 2.5 ml of Folin-Ciocalteu reagent and mixed well. 2.5 ml of 7.5% Na_2CO_3 was mixed after 5 min, and test tube was stand at 45ºC temperature for 45 min. Spectrophotometer (model 4001/4, Thermo Fisher Scientific) was used to measured absorbance at 765 nm. The calculation of phenolic amount was taken in three times and result was produced as average form. The content of phenolic in samples was showed in form of gallic acid equivalent (mg of Gallic acid/g of extract.[7]

2.6 Total flavonoids content

The flavonoid total amount was determined with little modifications.[8]1 ml/ml of methanolic extract solution was dissolved in 1 ml 2% alimunium-tri-chlorodesolution in CH_3OH. The sample was kept at room temperature for 1 hour. The absorbance was taken at wavelength 415 nm spectrophotometer (model 4001/4, Thermo Fisher Scientific).Triplicate samples were prepared and analyzed and average of absorbance was taken. The content of flavonoids in samples was showed inform of rutin equivalent (mg of Rutin/g of extract).

2.7 Free radical scavenging activity by DPPH

Freshly made0.2mM of DPPH (2,2-diphenyl-1- picrylhydrazyl) solution was added in sample dilutions (50-250 µg/ml) to eachtest-tube tillthat 3ml then after half hour, absorbance was taken at 517 nm by spectrophotometer. Ascorbic acid was taken as standard.The control was made as thesimilar without extract.

Percentage inhibition was measured by theformula.[9]

% inhibition of free radical scavenging potential

$$= \frac{(Control_{OD} - Sample_{OD})}{Control_{OD}} x100$$

2.8 Nitric oxide assay

Nitric oxide radical scavenging capacity measured through Griess Ilosvay reaction. At physiological pH 7.2 sodium nitroprusside decompose in aqueous medium and generates NO. In the presence of oxygen NO make nitrate and nitrite, both product are stable. According to protocol sodium nitroprusside (10 mM), saline phosphate buffer (0.5 ml) and ST extracts (50-250 µg/ml) was mixed and rested at 30°C for 120 min. After that 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2% H3P04 (Griess reagent)was mixed and kept10 min for completing the reaction diazotization. Then, naphthyl ethylene diaminedihydrochloride (1 ml) was mixed and kept for ½ hour sat 25°C. In diffused light a pink color chromophore is generated. The absorbance of this reaction mixture was observed at 540 nm wavelength. Rutin was taken as a standard.[10]

Formula

$$\%inhibition = \frac{(OD_{control} - OD_{Test})}{OD_{control}} x100$$

2.9 Metal chelating method

The ferrous level was observed by calculating the generation of the ferrous ion-ferrozine complex.1.0 ml of ST extracts, 0.1ml of 2 mM ferrous chloride and 5 mM ferrozine (0.2 ml) tostart the reaction and the mixture was shaken well and stand for 10 min. The absorbance of thesample was observed at 562 nm. Ascorbic acid taken as positive controls and analysis was run in triplicate.The chelating effect of Ferrozine-Fe2+ complexin percentage formation was calculated.[11]

$$\%ChelatingActivity = \frac{[(A_1 - A_2)}{A_0} x100$$

Where A_0 indicates the absorbance of the control and A_1indicates the absorbance of reaction mixture, A_2indicates the absorbance not including $FeCl_2$.

2.10 Antimicrobial potential with MIC

2.10.1 Agar well diffusion method

The antimicrobial activity *in-vitro* of ST all extracts was detected with thestandard agar well diffusion method.[12, 13]Potato Dextrose Agar and Mueller Hinton agar media were taken for antifungal and antibacterial activity. Petri plates poured 20 ml molten media medium were seeded by the fungal/bacterial strains inoculums (200µl, 1×10^8cfu/ml). The labeled media plates were stand to solidify uniformly and wells were puncheredwith the help of borer and 50-150µg/ml of the ST extracts dissolved in 100% DMSO was filled. Then left for incubated at 27°C and 37°C for 48 and 24 hours for fungi and bacteria respectively. For negative control Dimethyl sulfoxide (DMSO) was taken. Fluconazole and Streptomycin (10µg/disc) was taken as standard and reading was measured in triplicates. The antifungal and antibacterial activities were detected using the inhibition zone diameter formed around the well. The zone of inhibition diameter was calculated in millimeters (mm).

2.10.2 Minimum inhibitory concentration(MIC)

96-well culture plates were taken and two-fold serial dilutions of the aqueous and methanolic extracts(4-128µg/ml) were prepared in dimethyl sulfoxide (DMSO) for MIC of antifungal and antibacterial activity. The MIC of ST extracts which exhibited zones of inhibition significantly against test strains was determined. The MIC was described as the least concentration ability to stop any observable fungal or bacterial growth.[14]

2.11 Cytotoxic assessment

2.11.1 Human cell lines and culture

MTT (3-(4,5-Dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide, a tetrazole) protocol was used to estimate the anti-proliferative result of ST extracts. In this process, enough quantity of exponentially growing cells was taken to block\confluence of the culture media throughout the procedure. The cell lines HepG2, HCT-116, SW-60, A-549 and MD MBA-231 were seeded at 10^4cells/well and stand to incubated for 12 hours.

2.11.2 Cytotoxicity analysis

In sequence to estimate the desired amount of concentration at which the extracts showed proliferation in all testing cell lines, cells were treated with the extracts at a 100 µg/ml concentration. DMSO was taken as a solvent for the dissolving of extracts and it was also taken as blank. 5-Fluorouracilat a concentration of 1×10^{-5} µg/ml was taken as standard. Cell growth was observed after 48 h treatment according to MTT protocol.[15]50µl MTT solution (5 mg/ml of PBS) was mixed to each well and the 96-plates were incubated at 37°C for 3 hours in dark place. The culture media was aspirated and MTT solvent in amount of 150 µl (0.1% Nondet P-40, 4 mM HCl, all in isopropanol) was added in each well to dissolve the formazan crystals. The ELISA reader was used to measured absorbance at 570 nm of plates (Benchmark, Bio-Rad). All sample reading was taken in triplicate, and the whole procedure was repeated thrice.

3. Results And Discussion

3.1 Percentage extractive yield

The percentage yield by hot extraction of polyherbal formulation *Safoof-e-Tabkheer* (ST) using various solvent (Figure 1). The yield was highest in AqSt followed by MeSt extract. The extraction was done using increasing polarity order: pet-ether<chloroform<ethyl acetate<methanol and aqueous accordingly. The ability to extract phytoconsituents is more in ST the amount of methanolic and aqueous extracts was higher (28.85%, 19.42%). Extraction with pet-ether and chloroform showed the lowest yield (4.61%, 5.26%). Ethyl acetate has shown14.56% extractive value. On the basis of result it is concluded that more polar compound was extracted in MeSt extract.

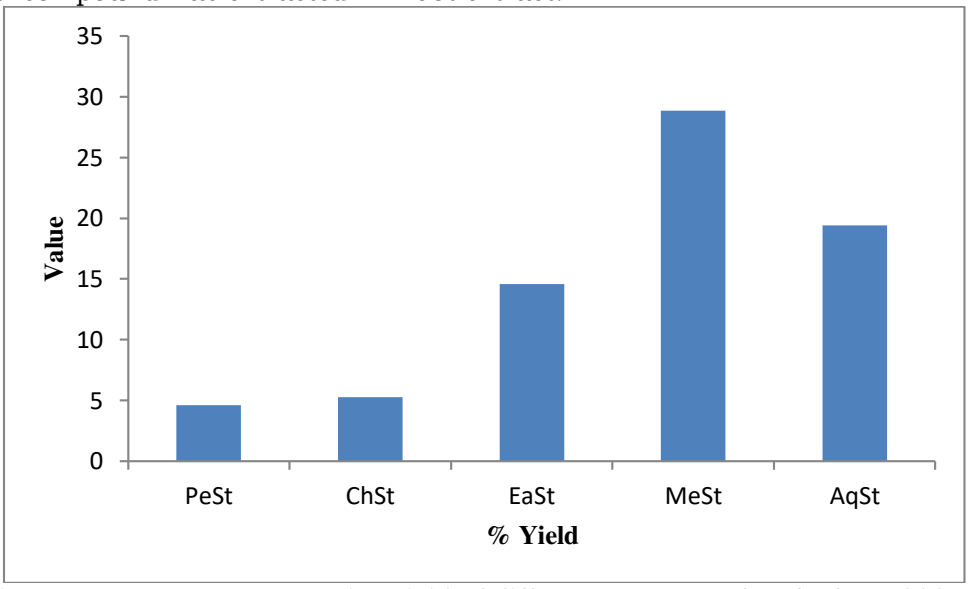


Figure 1: Percentage extractive yield of different extracts of *Safoof-e-Tabkheer*

3.2 Total Phenolic content and Flavonoid content

The health beneficial qualities related with flavonoids and phenolics compounds has important to quantify in herbal drug formulation and food products. The total amount of flavonoid and phenolic content shown in (Table 1)Gallic acid equivalent estimated higher range were 152.4±1.12, 194.7±1.15 mg/g in methanolic (MeSt) extract and 105.01±1.13, 149.4±1.64 mg/g in aqueous (AqSt) extracts of *Safoof-e-*

Tabkheer.(EaSt) shown 104.02±0.97 and 134.03±1.0moderate amount of flavonoid and phenolic content. The least amount of flavonoid and phenolic content estimated by ChSt (66.83±0.77 and 80.03±0.65) and PeSt (37.68±0.42 and 47.73±1.32) extract of ST (Figure 2).

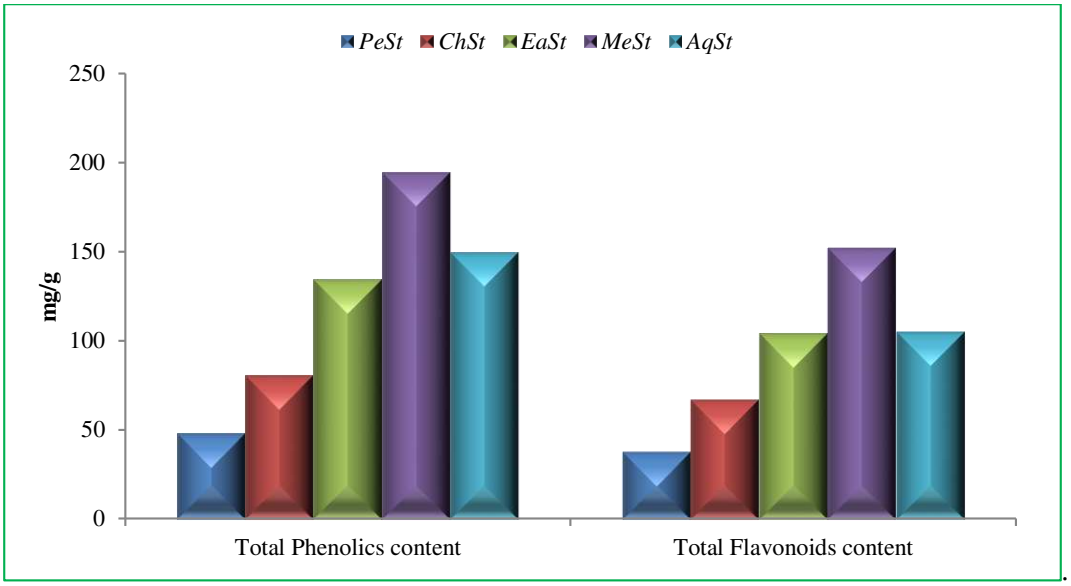


Figure 2: Total phenolic content and Total flavonoid content of ST extracts

Table 1: Total flavonoid and phenolics content of all extract of ST.

Extract	Total Phenolics content (mg GAE/g)	Total Flavonoids content (mg RU/g)
PeSt	47.73±1.32	37.68±0.42
ChSt	80.03±0.65	66.83±0.77
EaSt	134.03±1.0	104.02±0.97
MeSt	194.7±1.15	152.4±1.12
AqSt	149.4±1.64	105.01±1.13

Data expressed as mean ± SD, n=3;(a): average of three determinations

***PeSt*:** Pet ether$_{60\text{-}80}$ extract of *St*, ***ChSt*:** Chloroform extract of *St*, ***EaSt*:** Ethyl acetate extract of *St*, ***MeSt*:** Methanol extract of*St*, ***AqSt*:** Aqueous extract of *St*, ***St*:** *Safoof-e-Tabkheer.*

3.3 Frees radical scavenging (DPPH)capacity

The capability of aqueous, methanolic, ethyl acetate, chloroform and pet-ether extract of ST to scavenge 2,2-diphenyl-1- picrylhydrazyl) free radical were estimated in the form of % inhibition and IC_{50}87.14% (*IC50*=98.65±1.47µg/mL), 96.13% (*IC50*=82.31±2.16µg/mL),78.45% (*IC50*=127.23±2.26µg/mL),18.54%

(*IC50*=696.27±2.34µg/mL) and 07.24%(*IC50*=1538.34±2.30µg/mL) at 250µg/ml concentration, where ascorbic acid % inhibition and IC_{50}at the equal concentration was 98.09% (*IC50*=45.96±2.69µg/mL) (Figure 2).

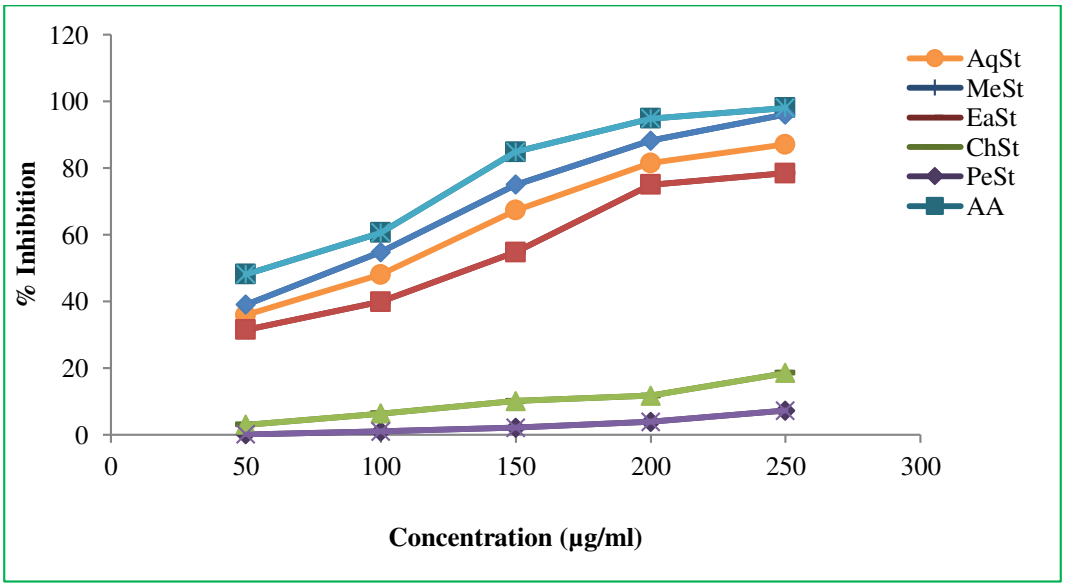


Figure 3: DPPH scavenging capacity of ST extracts

3.4 Metal chelating capacity

The metal chelating capacity of aqueous, methanolic, ethyl acetate, chloroform and pet-ether extract of ST were depend on concentration. The absorbance of Fe2+-ferrozine complex was concentration dependent so that it was linearly decreased. The metal chelating capacity percentage and IC_{50}at the 500 µg/ml concentration were found to be 87.51% (*IC50*=201.36±1.71), 80.07% (*IC50*=233.38±2.39), 75.54% (*IC50*=295.94±2.54), 61.81% (*IC50*=386.53±2.43) and 44.31% (*IC50*=593.79±2.63) that ascorbic acid for standard was found to be 96.54% (*IC50*=44.97±2.67) at same concentration (Figure 3).

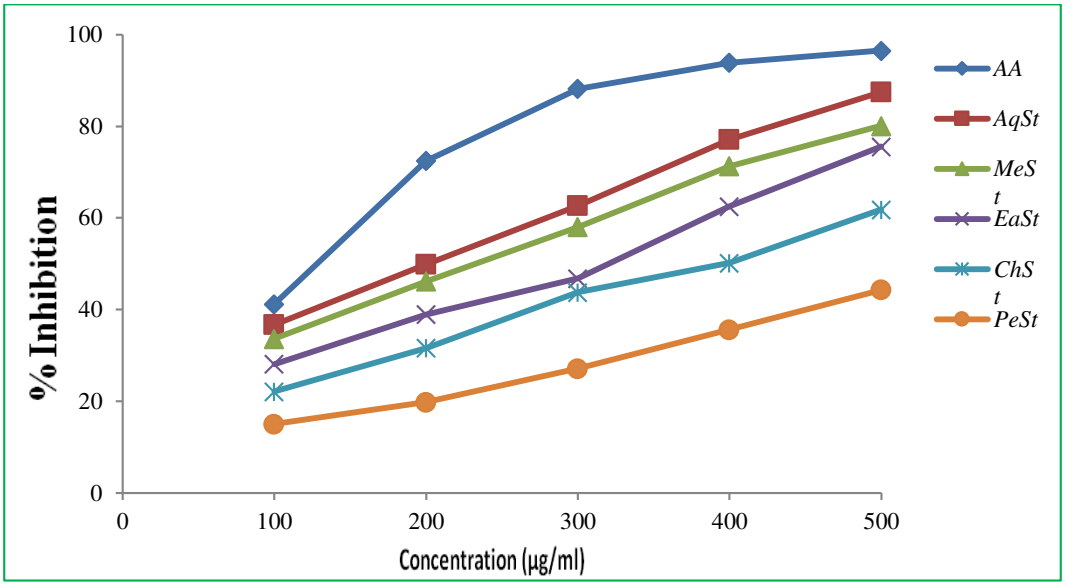


Figure 4: Metal chelating ability of ST extracts

3.5 Nitric oxide radical inhibition assay

The ability of aqueous, methanolic, ethyl acetate, chloroform and pet-ether extract of ST to Nitric oxide scavenge radical property was calculated by %inhibition were found 97.14 (*IC50*=72.14±1.04), 95.12 (*IC50*=84.28±1.54), 93.16 (*IC50*=124.58±2.10), 27.99 (*IC50*=483.10±1.02), and 19.32 (*IC50*=610.75±2.24), at 250µg/ml concentration, whereas standard rutin % inhibition at the equal concentration were 98.49(*IC50*=48.35±1.04), (Figure 5).

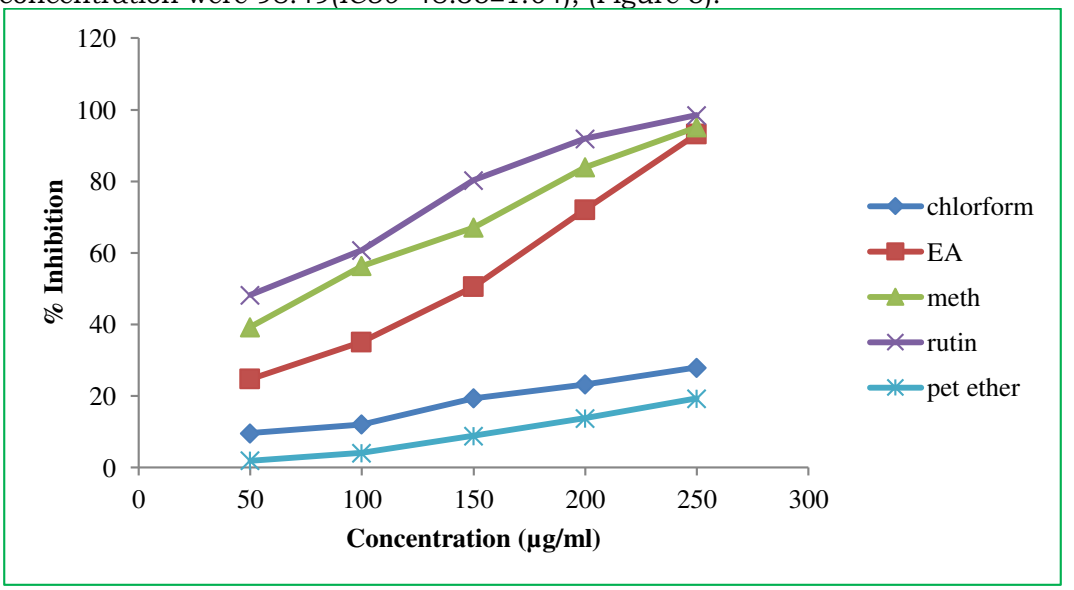
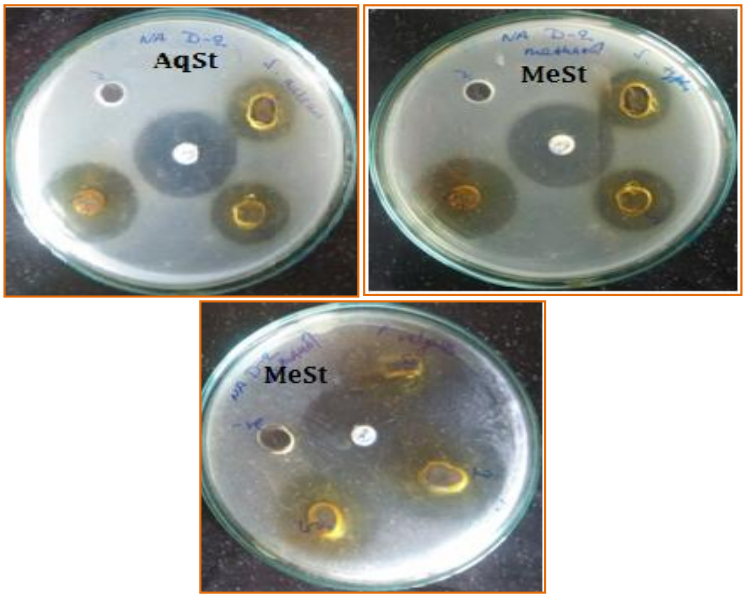


Figure 5: Nitric oxide radical scavenging ability of ST extracts

3.6 Antimicrobial activity with MIC

From last few years, drastic increment in the field of research for natural products with capacity of antimicrobial because they expect to discover novel compound with promising antimicrobial activity and low adverse effect for human health. Antimicrobial activities *in-vitro* in order to increase polarity extracts of ST and standard antibiotics against fungal and (gram negative and positive) bacterial strains with their MIC value of MeSt and AqStare given in Table 2 and 3. The AqSt and MeSt extract shows good inhibitory potential against all six bacterial strains and five fungal strains. EaSt and ChSt show moderate activities against all bacterial strains while PeSt shown least activity.



AqSt against a) *S. aureus*; *MeSt* against b) *S. typhi*and c) *P. valgaris;*

Figure 6: Images of Zones of inhibition in AqSt and MeSt extracts of *Safoof-e-Tabkheer*(*St*) using Streptomycin as Standard and DMSO as negative control against various bacterial strains.

Table 2: In-vitro antibacterial activity and MIC of Safoof-e-Tabasheer

Test bacteria	**Zone of inhibition (in mm)**					Zone of inhibition of antibiotic (mm)	**MIC(µg/ml)**	
	PeSt	*ChSt*	*EaSt*	*MeSt*	*AqSt*		*AqSt*	*MeSt*
Gram-Positive Bacteria								
S. aureus (MTCC 96)	17±0.03	15±0.34	22±0.13	30±0.37	30±0.11	30±0.50	16µg/ml	32µg/ml
K. pneumonia (MTCC 19)	15±0.31	22±0.21	26±0.23	30±0.65	30±0.67	25±0.45	32µg/ml	32µg/ml
Gram-Negative Bacteria								

P. vulgaris (MTCC 426)	14±0.47	18±0.56	26±0.15	30±0.17	30±0.0	29±0.28	64µg/ml	16µg/ml
S. typhi (MTCC 98)	19±0.69	18±0.48	30±0.15	30±0.26	32±0.0	30±0.73	8µg/ml	32µg/ml
E. coli (MTCC 739)	25±0.62	25±0.41	30±0.31	32±0.34	32±0.0	30±0.31	8µg/ml	64µg/ml
P. aeruginosa (MTCC 1688)	15±0.17	20±0.27	30±0.15	31±0.16	30±0.02	30±0.15	8µg/ml	32µg/ml

*Each Zone of inhibition (in mm) value represents the **Mean ± SD** of three experiments*

Table 3: In-vitro antifungal activity and MIC of Safoof-*e-Tabasheer*

Test fungus	**Zone of inhibition (mm)**					**Zone of inhibition of antifungal (mm)**	**MIC (µg/ml)**	
	PeSt	*ChSt*	*EaSt*	*MeSt*	*AqSt*		*AqSt*	*MeSt*
S. cerevisae MTCC 170	19±0.05	20±0.03	23±0.17	25±0.14	23±0.14	25±0.0	32	16
A. fumigatus MTCC 900	19±0.04	22±0.06	30±0.11	26±0.04	26±0.1	25±0.0	128	64
P. crysogenum MTCC 947	20±0.13	19±0.57	25±0.13	25±0.11	25±0.14	25±0.01	64	32
H. viridescens SITCC	22±0.0	24±0.08	23±0.19	25±0.06	25±0.0	25±0.0	64	32
M. plumbeus SITCC	19±0.13	24±0.04	22±0.0	25±0.10	24±0.03	25±0.04	64	32

*Each Zone of inhibition (mm) value represents the **Mean ± SD** of three experiments*

3.7 Cytotoxic assay

Cytotoxic experiment by MTT method using cultured A-549 (lung), MD MBA-231 (breast), HCT-116 (colon), HepG2 (hepato) and SW-60 (colon) cell lines in order to show the effect of ST extracts on human cancer cell lines (Table 4). MeSt was active against HCT-116 and A-549 cancer cell lines. The abundance of various phytochemicals in the MeSt and AqSt extracts is responsible for cytotoxic activity of *Safoof-e-Tabkheer.*

Table 4: IC_{50} value of different extracts of *Safoof-e-Tabkheer* (*St*) (*In-vitro*)

Cell lines					
Material	**(A-549)[a]**	**(HCT-116) [a]**	**(HepG2) [a]**	**(MD MBA-231) [a]**	**(SW-60) [a]**
5-Fluorouracil	1.33±1.2 µg	1.34±0.2 µg	0.93±1.3 µg	1.30±0.6 µg	1.49±2.2 µg
AqSt	**46.34±0.6 µg**	**65.61±1.1 µg**	80.11±1.2 µg	**44.4±0.7 µg**	**67.39±1.4 µg**
MeSt	90.14±1.1 µg	**47.84±2.0 µg**	>100 µg	>100 µg	>100 µg
EaSt	>100 µg	>100 µg	>100 µg	>100 µg	>100 µg
ChSt	NS	>100 µg	>100 µg	NS	>100 µg
PeSt	NS	>100 µg	NT	NT	NT

a *= IC_{50}; NS=Not shown any cytotoxicity to this cell line; NT=Not tested against this cell line. (All values are the mean ±SD of three replicates.)*

4. Conclusion

Based on the above mentioned results, it is concluded that the aqueous and methanolic extracts of *Safoof-e-Tabkheer* (ST) posses string antimicrobial, cytotoxic potential, antioxidant activity, evidenced by the free radical scavenging property, Metal chelating and Nitrous oxide scavenging which is on the basis ofthe presence of phenolic and flavonid phytoconsituents in the extract. The present investigationsuggests that the unani herbal formulation ST which possesses good antimicrobial and antioxidant potential is a better supplement for the diseases associated with oxidative stress.

Declaration

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Conflict of Interest None

Ethical approval none

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