ORIGINAL ARTICLE

Identification of polyphenols in leaf extracts of *Lawsonia inermis* L. with antioxidant, antigenotoxic and antiproliferative potential

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Background: Keeping in view the importance of natural plant products, bioactivity-guided isolation of phytoconstituents was attempted from *Lawsonia inermis* L. **Materials and Methods:** Various polar and non-polar extract/fractions were isolated and evaluated for the genotoxic and antigenotoxic potential against mutagens viz., 4-nitroquinoline-1-oxide and nitrofurantoin in SOS chromotest using *Escherichia coli* PQ37 tester strain and against H_2O_2 in DNA protection assay. In order to decipher mode of action, antioxidant activity was evaluated using different assays. The extract/fractions were also investigated for their antiproliferative potential against PC-3 and Colo 205 cancer cell lines. Major polyphenolic constituents were identified using high-performance liquid chromatography (HPLC) technique. **Results:** All the extract/fractions except Hex-Li significantly decreased the SOS-inducing potency of both the mutagens. In various *in vitro* assays, the polar fractions exhibited marked antioxidant activity. HPLC analysis of the extract/fractions showed that gallic acid, catechin, chlorogenic acid, epicatechin, rutin, ellagic acid, quercetin and kaempferol are the major constituents. The marked antigenotoxic activity of extract/fractions may be attributed to the polyphenolic compounds. The fractions viz., Hex-Li and $CHCl_3$-LI were found to be highly cytotoxic against Colo-205 cancer cell line. **Conclusions:** This is the first report of antigenotoxic and anticancer activities of *Lawsonia inermis* L. phytoconstituents. The antiradical potency of these extract/fractions may be responsible for the above activities.

Key words: 4-Nitroquinoline-1-oxide, antioxidant activity, cancer cell lines, nitrofurantoin, SOS chromotest

INTRODUCTION

In the last few years, numerous mechanistic studies on the cancer helped in the development of new chemopreventive agents. Chemopreventive agents provide protection by the mechanisms of antimutagenic, antioxidant and antiproliferative activity.[1] Antimutagens modulate the effect of mutagens and/or carcinogens and antioxidants scavenge reactive oxygen species, thus averting DNA damage. On the other side, antiproliferative agents induces apoptosis, halt cell cycle and DNA synthesis etc.[2] From last few decades, researchers all over the world are working on cancer prevention and management but still we have long way to go. Carcinogenesis is a multistep process and oxidative DNA damage has been suggested as a likely contributor to cancer etiology through several mechanisms.[3,4] Any mutation occurring due to the DNA damage caused by oxidants/genotoxins may lead to formation of cancerous tissue. The exposure to these environmental genotoxins cannot be avoided because of modern life style, thus ingestion of protective phytochemicals in the form of fruits/vegetables is the one of the best option to avoid cancer. The main mechanisms by which these chemopreventive agents can provide protection against cancer includes increasing detoxification and removal of gentoxins/mutagens from the body. These agents also possess the potential to act upon at any stage of carcinogenesis viz., intiation, promotion and progression,[5-8] thus leading to cancer chemoprevention.

Several environmental factors/agents including ionizing radiations, UV light, chemical compounds and physiological processes occurring in the body produce reactive oxygen species (ROS) as by products which in turn cause oxidative stress.[9] ROS including superoxide anions, hydroxyl radicals, hydrogen peroxide, alkoxyl radicals cause damage to the biological macromolecules (DNA, proteins, lipids, carbohydrates etc.) resulting in various degenerative diseases like cancer, atherosclerosis, cardiovascular diseases etc.[10-12] Besides, these ROS are also responsible for food deterioration during processing. ROS may result in rancidity in the food along with discoloration, vitamin destruction, aroma and nutritional losses etc.[13] Plant extracts are rich in natural antioxidants and antimutagenic agents and

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can be utilized for development of new drugs.[14] Further, the natural molecules are safer for consumption and may act as alternative for synthetic drugs. Reports from several studies suggest that plant-based diets have been and continue to be an important source of anticancer agents.[15,16] The plants with ethno-pharmacological use are of great importance in the search for active compounds.

Lawsonia inermis L. is a monotypic genus consisting of only one species. Thus, the plant is not only taxonomically important but also possesses numerous medicinal properties which needs to be explored. In India, it is commonly known as Mehndi/Henna and applied to hands, nails, hairs and feet as a natural dye. The different plant parts are used in traditional medicine system.[17-19] Keeping in mind, the role of medicinal plants in ameliorating oxidative stress critical in the malignant transformation of most types of cancer, the present study was aimed to assess antigenotoxic, antioxidant and anticancer activity of extract/fractions from *Lawsonia inermis* L.

MATERIALS AND METHODS

Plant Material

The leaves of the *L. inermis* L. were purchased from local market at Amritsar, Punjab, India and voucher specimen (no. 6773) has been submitted to the Herbarium of the Department.

Extraction and Isolation

The leaves (3 kg) were washed and cleaned with water to remove any dust impurities and dried in shade. They were ground and dipped in 80% methanol (4 × 6 litres) to obtain the methanol extract (MeOH-LI; 120 g). MeOH-LI was then dissolved in distilled water in a separating funnel and fractionated with organic solvents viz. hexane, chloroform, ethyl acetate and butanol to obtain Hex-LI (8.3 g), CHCl3-LI (10 g), EtOAc-LI (30.65 g), But-LI (25.5 g) respectively. The remaining aqueous fraction was named as AQ-LI (26.9 g). Finally all the fractions were concentrated and dried using rotary vacuum evaporator (Buchi Rotavapor R-210).

Phytochemical Analysis

Total Phenolic Content (TPC Assay)

The Folin-Ciocalteu method was used to determine total phenolic content (TPC) of extract/fractions.[20] The phenolic content was determined as gallic acid equivalents (GAE) in mg/g of dry sample.

Total Flavonoid Content (TFC Assay)

The total flavonoid content (TFC) is determined by using rutin as a standard.[21] The total flavonoid content was determined as rutin equivalents (RE) in mg/g of dry sample.

High-Performance Liquid Chromatography Analysis

Sample Preparation

For high-performance liquid chromatography (HPLC) analysis, samples were dissolved in methanol (HPLC grade) and filtered through a 0.22 µm Acrodisc® syringe filter (PALL, Life Sciences) and then injected into the UHPLC system.

HPLC Instrument and Chromatographic Conditions

HPLC analyses were performed on Shimadzu UHPLC Nexera system (Shimadzu, MA, USA). A photodiode array (PDA) detector was used for detection. An Enable C_{18} column (150 mm × 4.6 mm, i.d. 5 µm) was used with column temperature (25°C). Samples were separated using a gradient mobile phase consisting of 0.1% acetic acid aqueous as solution A and Methanol as solution B. The gradient elution is: 0-1 min, 30% B; 1-10 min, 65% B; 10-14 min, 80% B; 14-16 min, 80% A, 16-17 min: 40% B, 17-20 min: 35% B and 20-21 min: 30% B. The flow rate was set as 1 ml/min and the injection volume was 5 µl.

Antigenotoxicity Assay

Bacterial Tester Strain

Escherichia coli PQ37 strain purchased from Institut Pasteur, France, was used for antigenotoxicity study. Frozen permanent copies of the tester strain were prepared and stored at -80°C.

Genotoxicity Assay

The SOS chromotest with *E. coli* PQ37 strain was carried out by the method of Quillardet and Hofnung[22] with slight modifications. About, 1 ml of an overnight grown culture of *E. coli* PQ37 was diluted with 9 ml of Luria broth medium. About 600 µl of diluted bacterial culture was distributed to a series of glass test tubes followed by addition of mutagens viz. 4-nitroquinoline-1-oxide (4 NQO) (20 µg/assay)/ nitrofurantoin solution (NF) (10 µg/assay) and different concentration (10, 30, 100, 300 and 1000 µg/assay) of extract/ fractions. The mixtures were incubated with shaking for 2 h at 37°C. After incubation, 300 µl of each reaction mixture was used for assay of β-galactosidase and alkaline phosphatase activities, respectively, according to the standard protocol.[22] The absorbance of reaction mixture was measured at 420 nm against a blank without bacteria.

The β-gal and AP enzyme activities were calculated as:
Enzyme units (U) = $A_{420} \times 1000/t$
(A_{420} = optical density at 420 nm; t = substrate conversion time in minutes).
Induction Factor (IF) = Rc/Ro
Rc = β-galactosidase activity/alkaline phosphatase activity determined for the test compound at concentration c,
Ro = β-galactosidase activity/alkaline phosphatase activity in the absence of the test compound.

Compounds are treated as non-genotoxic if the induction factor (IF) remains <1.5, as slightly genotoxic if the induction factor ranges between 1.5 and 2 and as genotoxic if the IF exceeds 2.[23]

Antigenotoxicity was expressed as percentage inhibition of genotoxicity induced by 4-NQO/NF according to the formula:
Inhibition (%) =100 − (IF1 − IF0/IF2 − IF0) × 100

Where,
IF_1 is the induction factor of the test compound and the genotoxin
IF_2 is the induction factor of positive control (4NQO/NF)
IF_0 the induction factor of the blank (without any test compound).

DNA Protection Assay

DNA protection assay was performed using supercoiled pBR322 plasmid DNA with slight modifications.[24] Plasmid DNA was incubated with extract/fractions (50-250 µg/ml) alone to check genotoxicity of extract/fractions. In order to evaluate antigenotoxic ability, extract/fractions were added to reaction mixture containing pBR322 DNA and Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$). The mixture was then incubated for 30 min at 37°C followed by electrophoresis. Finally DNA was analyzed using Gel Doc XR system (Bio-Rad, USA). Percentage amount of DNA in different bands was determined on the basis of integrated density value using AlphaEase FC software.

Antioxidant Assays

Nitric Oxide Radical Scavenging

Nitric oxide radical scavenging was carried out by the method given by Marcocci *et al*.,[25] with slight modifications. The absorbance of the pink chromophore was read at 546 nm. Rutin was used as antioxidant standard.

Radical scavenging activity % = $A_0 - A_1/A_0 \times 100$

Where, A_0 is the absorbance of control (reaction mixture without test sample),
A_1 is the absorbance of reaction mixture containing test sample.

$ABTS^{+}$ Radical Scavenging Assay

$ABTS^{+}$ scavenging assay was carried out by the method given by Re *et al*.,[26] with slight modifications. The absorbance was taken up to 5 min at 734 nm (Systronics 2202 UV–Vis Spectrophotometer, India). Rutin was used as antioxidant standard.

Radical scavenging activity % = $A_0 - A_1/A_0 \times 100$

Where, A_0 is the absorbance of ABTS radical cation solution,
A_1 is the absorbance of reaction mixture.

DPPH Radical Scavenging Assay

DPPH scavenging activity was carried out by the method of Blois[27] with slight modifications. The absorbance of reaction mixture was taken at 517 nm using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). Rutin was used as antioxidant standard.

Radical scavenging activity % = $A_0 - A_1/A_0 \times 100$

Where, A_0 is the absorbance of DPPH solution,
A_1 is the absorbance of reaction mixture.

Ferric Ion Reduction Potential Assay

Reducing ability of extract/fractions was determined using the method of Oyaizu.[28] The absorbance was measured spectrophotometrically at 700 nm using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). The percentage of reduction of the sample as compared to the standard (Rutin) was calculated using the formula

% Reducing power = $[1 - (1 - A_s/A_c) \times 100]$

Where, A_c = absorbance of standard compound at maximum concentration tested, and A_s = absorbance of sample.

Superoxide Anion Radical Scavenging Assay

The superoxide anion scavenging activity was performed as per the method described by Nishikimi *et al*.,[29] with slight modifications. The reaction mixture was read at 560 nm using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). Rutin was used as the reference compound.

Antioxidant activity % = $A_0 - A_1/A_0 \times 100$

Where, A_0 is the absorbance of control
A_1 is the absorbance of reaction mixture containing test sample.

Total Antioxidant Capacity Assay

The antioxidant capacity of the sample was evaluated by the phosphomolybdenum method.[30] The absorbance of a solution was measured at 695 nm against a blank. Total antioxidant activity was expressed in comparison to ascorbic acid and calculated by following formula:

% TAC = $A_s - A_c/A_{aa} - A_c \times 100$

Where, A_c is the absorbance of the control (without extract),
A_s is the absorbance in the presence of the extract,
and A_{aa} is absorbance in the presence of ascorbic acid.

Antiproliferative Activity

Cell Culture

The human colon carcinoma (Colo-205) and prostate cancer (PC-3) cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C (humified) and 5% CO_2 atmosphere.

Cytotoxicity Assay

The colorimetric MTT assay was used to evaluate cytotoxic effects of *L. inermis* extract/fractions.[31] About 1 × 10^4 cells were seeded per well of the plate (100 ul) in the 96-well plate and incubated for 24 h in CO_2 incubator. Cells were treated with varying concentrations of extract/fractions for 24 h and after then the cells were treated with MTT (10 µl) per well of the plate and incubated for 2 h. Thereafter, the media was removed from the wells very carefully. Then DMSO (100 µl) was added per well of the plate to dissolve the crystals and finally the plate was read at 570 nm.

Statistical Analysis

The results were expressed as the mean ± standard error. Regression analysis was carried out by best fit method and IC_{50} values were calculated using regression equation. The significance of results was checked at $^*P \leq 0.05$.

RESULTS

Phytochemical Analysis

Total Phenolic Content (TPC Assay)

Different extract/fractions showed TPC in the order: EtOAc-LI (190.22 mg GAE per gram of dry extract) > But-LI (141.05 mg GAE per gram of dry extract) > MeOH-LI (125.09 mg GAE per gram of dry extract) > $CHCl_3$-LI (106.2 mg GAE per gram of dry extract) > Hex-LI (89.5 mg GAE per gram of dry extract) > AQ-LI (88.78 mg GAE per gram of dry extract) [Figure 1].

Total Flavonoid Content (TFC Assay)

It was observed that the total flavonoid content (TFC) was in the order: EtOAc-LI (188.16 mg RE per gram of dry extract) > But-LI (113.5 mg RE per gram of dry extract) > MeOH-LI (107.22 mg RE per gram of dry extract) > $CHCl_3$-LI (61.5 mg RE per gram of dry extract) > AQ-LI (16.5 mg RE per gram of dry extract) [Figure 1].

Identification of Phytoconstituents

HPLC analysis showed the presence of different polyphenolic compounds in the various extract/fractions. MeOH-LI, EtOAc-LI, But-LI contains gallic acid, catechin, chlorogenic acid, epicatechin, rutin, ellagic acid, quercetin and kaempferol in major quantity [Table 1 and Figure 2]. EtOAc-LI showed high concentration of gallic, catechin, chlorogenic acid, ellagic acid, rutin and kaempferol [Figure 2]. This fraction was found to be highly antigenotoxic and antioxidant. Hex-Li and $CHCl_3$ showed low concentration of these compounds; further these samples have less antioxidant activity. However, catechin, epicatechin, rutin, quercetin, kaempferol, caffeic acid and coumaric acid were absent in Hex-Li. Umbelliferone, coumaric acid, rutin, quercetin, caffeic acid and kaempferol were absent in AQ-LI fraction [Table 1].

Genotoxicity Assay

Results revealed that incubation of extract/fractions of *L. inermis* with bacteria *E. coli PQ37* did not show any toxicity. Different extract/fractions were found to be non-genotoxic at the tested concentrations as the induction factor remained less than 1.5 [Tables 2-7]. Further, it was also ascertained that there was no significant change in the alkaline phosphatase units in all the experiments carried out which is marker for the normal protein synthesis in *E. coli PQ37*. The results revealed that as the extract/fractions did not induce the SOS response, indicating that they did not produce any DNA lesions. All the extract/fractions were classified as non-genotoxic.

Antigenotoxicity Assay

Doses of 20 µg/assay of 4-nitroquinoline-1-oxide (4-NQO) and 10 µg/assay of Nitrofurantoin (NF) which induced a significant

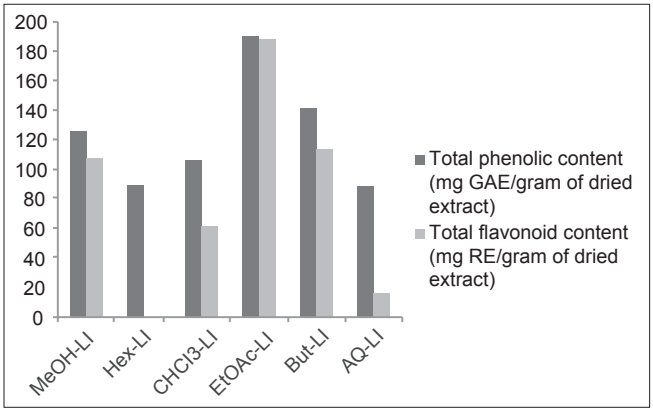

Figure 1: Total phenolic content and total flavonoid content of extract/fractions of *Lawsonia inermis* leaves

Table 1: Percent amount of different phytochemicals quantified by HPLC in different extract/fractions of *L. inermis* leaves

Extract/ fractions	MeOH-LI	Hex-LI	$CHCl_3$-LI	EtOAc-LI	But-LI	AQ-LI
Gallic acid	0.237	0.031	0.118	0.724	0.324	0.273
Catechin	0.701	-	0.078	1.566	1.287	0.101
Chlorogenic acid	2.203	0.047	0.328	1.738	1.211	-
Epicatechin	0.038	-	-	0.169	0.219	-
Caffeic acid	0.070	-	0.135	-	0.045	-
Umbelliferone	-	0.036	0.281	0.165	0.037	-
Rutin	0.210	-	0.073	0.791	0.045	-
Ellagic acid	1.741	0.231	0.911	5.929	1.070	0.826
Quercetin	0.161	-	0.390	0.412	0.128	-
Kaempferol	0.758	-	1.322	3.985	0.636	-
Coumaric acid	0.001	-	0.015	0.003	0.003	-

HPLC – High-performance liquid chromatography

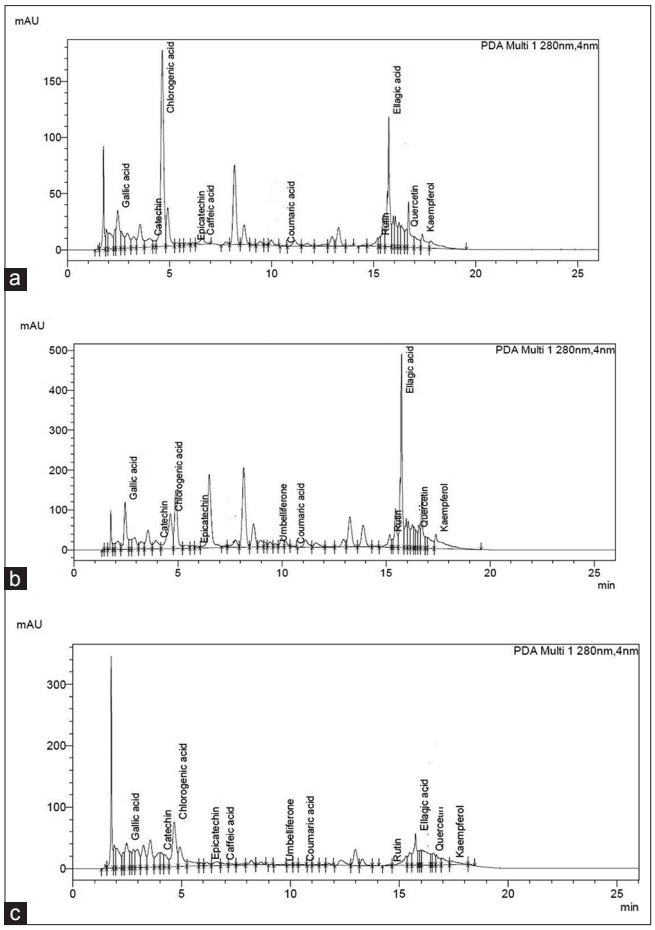

Figure 2: HPLC chromatograms of active extract/fractions (a) MeOH-LI, (b) EtOAc-LI and (c) But-LI of Lawsonia inermis leaves

SOS response (non-toxic concentration) in *E. coli PQ37* were selected to study the protective effects of *L. inermis* extract/fractions. From the Tables 2-7, it was clear that all the extract/fractions possess good potential to ameliorate the DNA damage. They decreased the induction factor induced by 4-NQO and NF. Different extract/fractions reduced the genotoxicity induced by 4-NQO in the order: $CHCl_3$-LI (55.30%) > EtOAc-LI (52.76%) > MeOH-LI (51.44%) > AQ-LI (48.89%) > But-LI (46.37%) > Hex-LI (9.08%). In the case of NF, the order of antigenotoxicity was But-LI (88.68%) > AQ-LI (87.34%)> EtOAc-LI (78.42%) > MeOH-LI (68.50%) > $CHCl_3$-LI (51.22%) > Hex-LI (37.80%). Further it was noticed that antigenotoxic potency of the extract/fractions was more against nitrofurantoin (NF) as compared to 4-nitroquinoline-1-oxide (4-NQO). The non-polar fraction i.e. Hex-Li was found to be least effective against both the genotoxins.

DNA Protection Assay

Hydroxyl radical generated by Fenton's reagent attacks supercoiled pBR322 DNA producing single stranded (resulting in nicked circular form II) or double-stranded breaks (resulting in linear form III). It was observed that plant extracts itself did not cause any DNA damage. The addition of different concentrations (50, 100, 150, 200 and 250 µg/ml) of various extract/fractions along with Fenton's reagent provided protection to pBR322 plasmid DNA [Figures 3-8]. DNA protective activity at the highest concentration for various extract/fractions was in the order: But-LI (100%) > EtOAc-LI (97.60%) > MeOH-LI (96.50%) > AQ-LI (92.90%) > $CHCl_3$-LI (92.80%) > Hex-LI (87.20%) [Table 8].

Table 2: Effect of MeOH-LI extract of *L. inermis* on genotoxicity of 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	63.13±1.910	46.18±1.159	8.85	-
NF	10	59.66±0.545	38.13±1.299	4.29	-
Negative control	0	8.40±0.251	54.13±3.740	1.00	-
	10	4.60±1.240	44.06±3.075	0.67	-
	30	4.30±0.081	39.46±3.326	0.69	-
	100	4.66±0.240	41.80±1.951	0.71	-
	300	4.00±0.100	35.53±1.219	0.72	-
	1000	5.35±0.384	34.76±2.154	0.98	-
4NQO+MeOH-LI	10	35.66±2.650	38.86±2.263	5.91	37.38
	30	34.23±2.816	39.33±3.433	5.61	41.25
	100	31.60±2.954	39.06±2.750	5.67	40.42
	300	31.81±0.033	38.50±2.845	5.32	44.94
	1000	36.06±0.779	48.33±3.707	4.81	51.44
					*r=0.9549
NF+MeOH-LI	10	26.66±0.762	33.36±0.819	2.19	63.74
	30	26.93±3.770	33.86±0.211	2.18	64.07
	100	26.86±0.883	34.66±1.203	2.12	65.83
	300	26.66±1.040	34.46±0.348	2.12	65.93
	1000	26.33±0.841	35.50±0.888	2.02	68.50
					*r=0.9534

*$P \leq 0.05$. NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin; SE – Standard error

Table 3: Effect of Hex-LI fraction of *L. inermis* on genotoxicity induced by 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	44.86±0.868	16.03±0.066	10.10	-
NF	10	78.30±0.763	51.73±0.982	4.74	-
Negative control	0	4.76±0.215	17.20±0.723	1.00	-
	10	4.43±1.153	15.80±0.513	1.01	-
	30	4.90±0.288	15.76±0.811	1.11	-
	100	4.73±0.066	16.30±0.793	1.04	-
	300	5.36±0.517	14.30±1.594	1.28	-
	1000	5.56±0.578	17.13±0.927	1.16	-
4NQO+Hex-LI	10	28.70±2.079	13.33±0.920	7.77	25.61
	30	30.06±3.860	13.13±0.952	8.26	20.22
	100	25.32±2.140	13.83±0.938	6.06	38.42
	300	33.23±2.560	13.30±0.510	9.01	11.92
	1000	35.73±1.980	13.90±0.569	9.27	9.08
					r=0.6663
NF+Hex-LI	10	51.47±0.762	44.40±0.680	3.63	29.59
	30	43.63±0.883	45.51±2.281	3.00	46.44
	100	40.90±3.770	45.56±0.440	2.81	51.57
	300	43.33±1.040	48.36±2.225	2.80	51.87
	1000	53.36±0.993	50.26±4.480	3.32	37.80
					r=0.3507

NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin

Table 4: Effect of CHCl3-LI fraction of *L. inermis* on genotoxicity induced by 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	56.33±1.485	15.93±0.218	9.90	-
NF	10	78.30±0.763	51.73±0.982	4.74	-
Negative control	0	5.66±0.317	15.83±0.145	1.00	-
	10	6.53±0.145	16.53±0.143	1.09	-
	30	6.06±0.284	17.66±0.260	0.96	-
	100	5.70±0.152	17.66±0.617	0.90	-
	300	6.06±0.145	18.25±1.295	0.92	-
	1000	6.63±0.240	17.00±0.602	1.05	-
4NQO+$CHCl_3$-LI	10	31.46±3.311	13.69±0.406	6.43	38.94
	30	29.43±1.970	13.56±0.145	6.07	42.96
	100	27.61±2.745	14.46±0.548	5.34	51.17
	300	30.56±1.010	15.03±0.504	4.87	56.53
	1000	28.36±0.633	15.93±0.968	4.98	55.30
					*r=0.9455
NF+$CHCl_3$-LI	10	46.80±1.501	45.23±0.837	3.29	40.08
	30	48.23±1.790	46.20±0.896	3.27	39.25
	100	36.53±1.396	46.83±1.835	2.44	61.36
	300	37.43±0.666	47.50±0.832	2.47	60.69
	1000	41.80±0.993	46.36±4.480	2.82	51.22
					r=0.8136

*$P \leq 0.05$. NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin

Antioxidant Assays

Nitric Oxide Radical Scavenging Assay

In this assay, the activity of extract/fractions based on percent inhibition was in the order: AQ-LI (60.47%) > EtOAc-LI (54.87%) > $CHCl_3$-LI (32.77%) > But-LI (30.83%) > MeOH-LI (16.08%). Hex-LI did not show any inhibition of NO radicals [Figure 9]. The results were compared with the standard flavonoid rutin (60.96%).

Table 5: Effect of EtOAc-LI fraction of *L. inermis* on genotoxicity induced by 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	43.20±0.680	19.40±0.817	8.24	-
NF	10	59.66±0.545	38.13±1.299	4.29	-
Negative control	0	6.93±0.352	25.60±0.145	1.00	-
	10	7.03±0.233	16.53±0.143	1.09	-
	30	7.00±0.100	17.66±0.260	0.96	-
	100	6.96±0.133	17.66±0.617	0.90	-
	300	6.50±0.378	18.25±1.295	0.92	-
	1000	7.90±0.493	17.00±0.602	1.05	-
4NQO+EtOAc-LI	10	26.43±1.166	18.26±0.520	5.35	39.82
	30	28.47±1.073	19.93±0.933	5.28	40.90
	100	25.33±1.637	17.90±1.650	5.24	41.46
	300	26.43±0.166	18.40±1.058	5.11	43.15
	1000	29.53±0.953	24.73±4.601	4.42	52.76
					*r=0.9959
NF+EtOAc-LI	10	27.33±0.809	37.03±0.584	2.02	68.84
	30	26.33±2.450	38.00±1.365	1.90	72.66
	100	25.30±1.138	37.56±0.823	1.84	74.27
	300	25.16±0.501	40.80±1.353	1.69	79.00
	1000	25.06±0.874	40.23±1.329	1.71	78.42
					*r=0.9523

*$P \leq 0.05$. NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin

Table 6: Effect of But-LI fraction of *L. inermis* on genotoxicity induced by 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	49.42±0.340	37.36±1.270	5.61	-
NF	10	59.66±0.545	38.13±1.299	4.29	-
Negative control	0	8.70±0.208	36.96±1.083	1.00	-
	10	7.70±0.450	38.63±0.635	0.87	-
	30	8.03±0.638	37.36±1.185	0.95	-
	100	7.10±0.057	39.46±1.105	0.95	-
	300	7.33±0.170	39.86±1.155	0.90	-
	1000	9.03±0.145	38.13±0.498	1.07	-
4NQO+But-LI	10	34.40±1.150	31.96±0.497	4.57	22.38
	30	28.00±1.361	32.20±1.178	3.69	41.49
	100	28.66±1.240	30.13±0.823	3.96	35.77
	300	28.13±1.230	30.60±1.209	3.91	36.87
	1000	28.51±0.590	34.90±1.677	3.47	46.37
					r=0.7589
NF+But-LI	10	26.83±1.844	33.73±0.405	2.18	64.07
	30	24.10±0.404	33.33±0.371	1.98	70.08
	100	22.20±1.210	35.20±1.978	1.73	77.85
	300	22.16±1.160	37.13±1.449	1.62	81.89
	1000	19.11±4.056	35.20±1.150	1.37	88.68
					*r=0.9969

*$P \leq 0.05$. NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin

ABTS Assay

In the present study, all the extract/fractions showed the potential to scavenge the $ABTS^{+}$ radicals. Their activity based on IC_{50} values was in the order: EtOAc-LI (IC_{50} 1.29 µg/ml) > MeOH-LI (IC_{50} 1.43 µg/ml) > But-LI (IC_{50} 2.90 µg/ml) > $CHCl_3$ (IC_{50} 27.24 µg/ml) > Hex-LI (IC_{50} 174.3 µg/ml) > AQ-LI (219.47 µg/ml) [Figure 10]. All the extract/fractions showed more than 95% inhibition at the highest tested concentration. The results were compared with the standard compound rutin (IC_{50} 24.78 µg/ml).

Table 7: Effect of AQ-LI fraction of *L. inermis* on genotoxicity induced by 4NQO (20 µg/assay) and NF (10 µg/assay) in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (µg/assay)	Mean±SE (units)		Induction factor	Percent inhibition
		β-Galactosidase	Alkaline phosphatase		
4NQO	20	86.03±1.219	31.13±2.587	19.60	-
NF	10	59.66±0.545	38.13±1.299	4.29	-
Negative control	0	4.56±0.866	32.36±2.722	1.00	-
	10	4.70±0.173	38.10±1.850	0.87	-
	30	4.63±0.188	34.36±0.788	0.95	-
	100	4.53±0.296	34.06±0.895	0.95	-
	300	4.33±0.066	33.96±1.128	0.90	-
	1000	5.20±0.750	34.53±0.920	1.07	-
4NQO+AQ-LI	10	58.96±1.283	33.50±1.537	12.57	37.81
	30	58.10±0.983	31.80±0.838	12.35	38.96
	100	55.00±1.008	35.03±0.841	11.21	45.10
	300	56.73±1.405	34.93±1.277	11.51	43.47
	1000	51.30±0.600	34.83±0.766	10.51	48.89
					*r=0.9338
NF+AQ-LI	10	26.23±1.464	40.20±0.587	1.79	75.84
	30	25.83±0.966	39.93±3.000	1.76	76.94
	100	23.16±1.138	33.93±1.266	1.87	73.51
	300	21.43±2.520	42.46±2.493	1.38	88.39
	1000	21.93±0.581	42.46±1.301	1.41	87.34
					r=0.7803

*$P \leq 0.05$. NQO – Nitroquinoline-1-oxide; NF – Nitrofurantoin

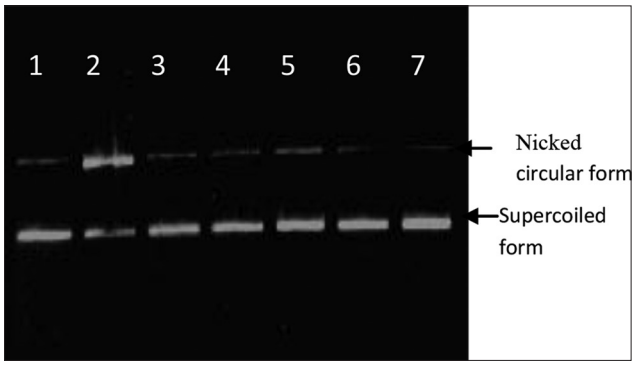

Figure 3: Effect of MeOH-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + MeOH-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + MeOH-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + MeOH-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + MeOH-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + MeOH-LI (250 µg/ml)

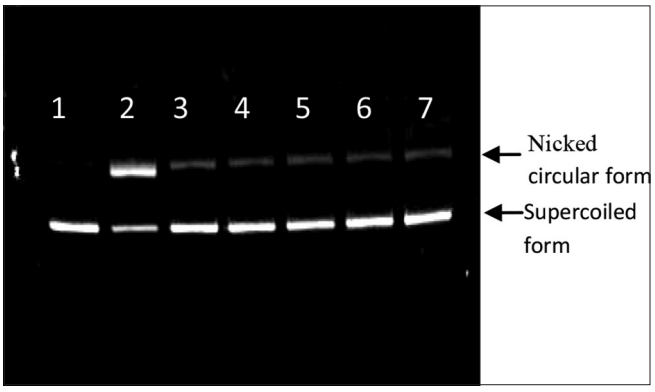

Figure 4: Effect of Hex-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + Hex-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + Hex-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + Hex-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + Hex-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + Hex-LI (250 µg/ml)

DPPH Assay

In DPPH assay, the antioxidants are able to reduce the DPPH radical to the yellow-coloured diphenylpicrylhydrazine. The order of antioxidant activity on the basis of their IC_{50} values was in the order: EtOAc-LI (IC_{50} 44.96 µg/ml) = MeOH-LI (IC_{50} 44.96 µg/ml) > But-LI (IC_{50} 90.01 µg/ml) > $CHCl_3$-LI (IC_{50} 252.13 µg/ml) > Hex-LI (IC_{50} 267.24 µg/ml) > AQ-LI (749.94 µg/ml) [Figure 11]. The AQ-LI showed least activity. Results were compared with rutin (IC_{50} 54.54 µg/ml).

Ferric Ion Reduction Potential Assay

Our results showed that out of different extract/fractions, EtOAc-LI showed highest reducing ability followed by But-LI, $CHCl_3$-LI and MeOH-LI. Hex-LI and AQ-LI were found to be least active and didn't achieve 50% inhibition even at highest tested concentration. The results were given in comparison to standard rutin. The reducing potential based on IC_{50} values was in the order: EtOAc-LI (IC_{50} 486 µg/ml) > But-LI (IC_{50} 504.76 µg/ml) > $CHCl_3$-LI (IC_{50} 782.49 µg/ml) > MeOH-LI (IC_{50} 900.83 µg/ml) [Figure 12].

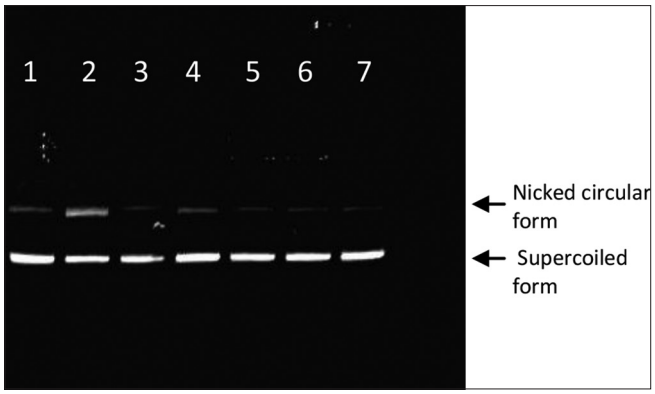

Figure 5: Effect of CHCl3-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + CHCl3-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + CHCl3-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + CHCl3-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + CHCl3-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + CHCl3--LI (250 µg/ml)

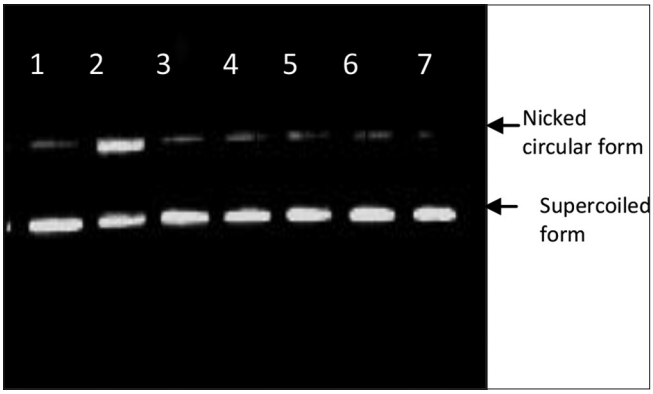

Figure 6: Effect of EtOAc-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + EtOAc-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + EtOAc-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + EtOAc-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + EtOAc-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + EtOAc-LI (250 µg/ml)

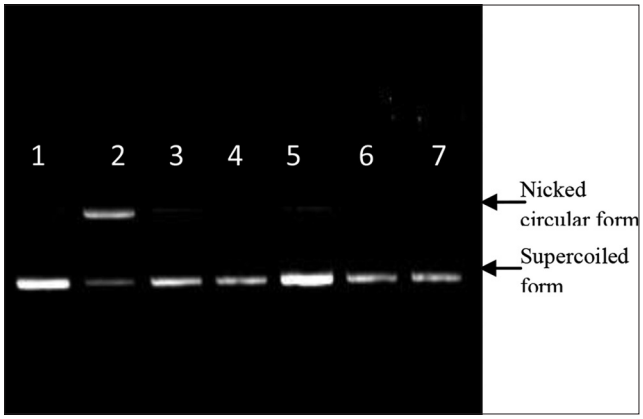

Figure 7: Effect of But-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + But-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + But-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + But-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + But-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + But-LI (250 µg/ml)

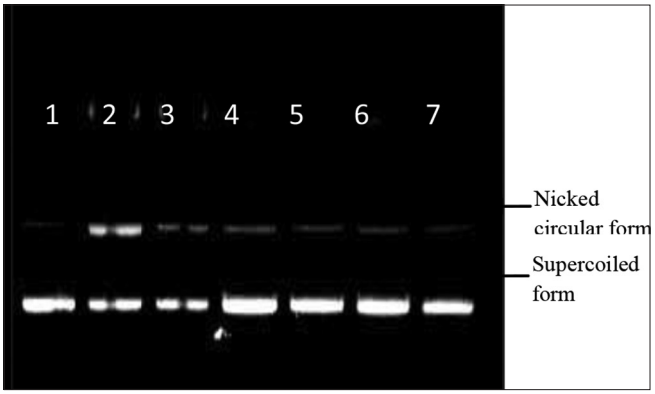

Figure 8: Effect of AQ-LI on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the Fenton's reagent. Lane 1: pBR322 DNA, Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control), Lane 3: pBR322DNA + Fenton's reagent + AQ-LI (50 µg/ml), Lane 4: pBR322 DNA + Fenton's reagent + AQ-LI (100 µg/ml), Lane 5: pBR322 DNA + Fenton's reagent + AQ-LI (150 µg/ml), Lane 6: pBR322 DNA + Fenton's reagent + AQ-LI (200 µg/ml), Lane 7: pBR322 DNA + Fenton's reagent + AQ-LI (250 µg/ml)

Superoxide Anion Radical Scavenging Assay

Our results showed that out of the six extract/fractions, only the polar fractions have the potential to scavenge superoxide radicals. The IC_{50} values were in the order: AQ-LI (IC_{50} 27.56 µg/ml) > MeOH-LI (IC_{50} 232.75 µg/ml) > But-LI (IC_{50} 235.56 µg/ml) > EtOAc-LI (IC_{50} 256.02 µg/ml) [Figure 13]. The non-polar fractions did not show any superoxide scavenging. Rutin showed IC_{50} of 58.75 µg/ml.

Total Antioxidant Activity

In the present study, all the extract/fractions reduced Mo(VI) to Mo(V) and the total antioxidant capacity of extract/fractions is expressed in comparison to ascorbic acid. The order of total antioxidant capacity was EtOAc-LI (27.5%) > But-LI (26.98%) > MeOH-LI (20.39%) > AQ-LI (17.17%) > $CHCl_3$-LI (16.31%) > Hex-LI (15.6%) [Figure 14].

Antiproliferative Activity

To investigate the inhibitory effects of extract/fractions on proliferation of PC-3 and Colo-205 cells, the cells were treated with different concentrations of extract/fractions for 24 h. Results obtained were very interesting as it has been observed that extract/fractions selectively showed antiproliferative activity only in Colo-205 cell line and were ineffective against PC-3 cell line. The maximum antiproliferative activity at highest tested concentration was in order: Hex-LI (80.82%) > $CHCl_3$-LI (59.07%) > MeOH-LI (58.85%) > EtOAc-LI (47.62%) > AQ-LI (23.93%). But-LI was found to be in effective in both cell lines.

Table 8: Percentage DNA calculated on the basis of integrated density value after treatment of pBR322 plasmid DNA with Fenton's reagent and different concentrations (50-250 µg/ml) of extract/fractions of *L. inermis* (using AlphaEase FC software)

Extract/ fractions	Form	pBR322 DNA	Fenton's reagent+ pBR322 DNA	pBR322 DNA+Fenton's reagent+Concentration of extract/fractions (µg/ml)				
				50	100	150	200	250
MeOH-LI	Open circular form (II)	11.20	66.10	10.00	10.20	16.90	5.90	3.50
	Supercoiled form (I)	88.80	33.90	90.00	89.80	83.10	94.10	96.50
Hex-LI	Open circular form (II)	2.20	67.80	16.70	12.50	15.00	11.40	12.80
	Supercoiled form (I)	97.20	32.20	83.30	87.50	85.00	88.60	87.20
$CHCl_3$-LI	Open circular form (II)	14.10	38.90	10.50	13.80	9.10	7.50	7.20
	Supercoiled form (I)	85.90	61.10	89.50	86.20	90.90	92.50	92.80
EtOAc-LI	Open circular form (II)	11.50	51.80	11.30	14.00	12.00	9.40	2.40
	Supercoiled form (I)	88.50	48.20	88.70	86.00	88.00	90.60	97.60
But-LI	Open circular form (II)	0	73.20	8.70	10.20	0	0	0
	Supercoiled form (I)	100	26.80	91.30	89.80	100	100	100
AQ-LI	Open circular form (II)	5.40	36.60	20.80	13.71	10.00	9.60	7.10
	Supercoiled form (I)	94.60	63.40	79.20	86.30	90.00	90.40	92.90

DNA – Deoxyribonucleic acid

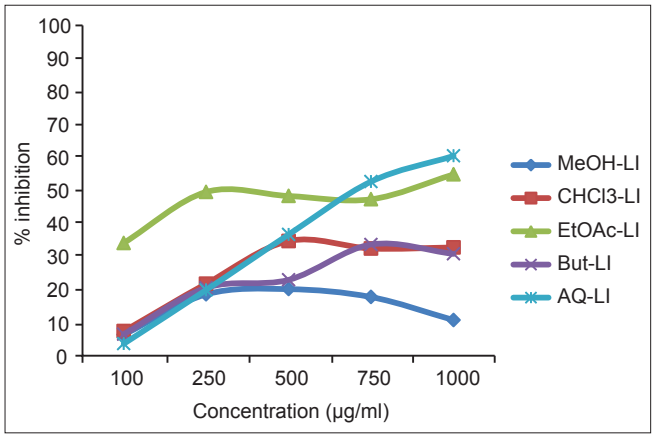

Figure 9: Scavenging effects of extract/fractions of *Lawsonia inermis* leaves on NO radicals

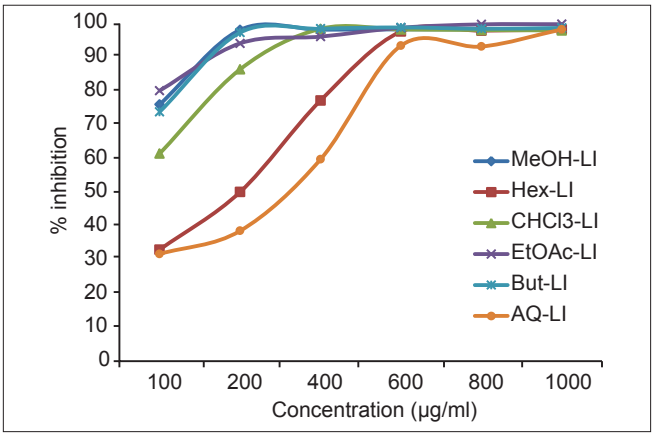

Figure 10: Scavenging effects of extract/fractions of *Lawsonia inermis* leaves on ABTS radicals

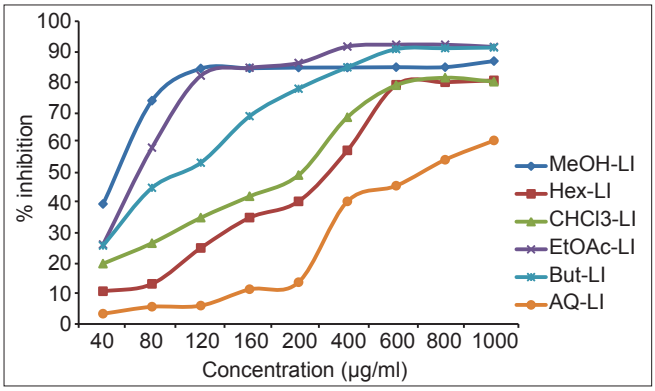

Figure 11: Scavenging effects of extract/fractions of *Lawsonia inermis* leaves on DPPH radicals

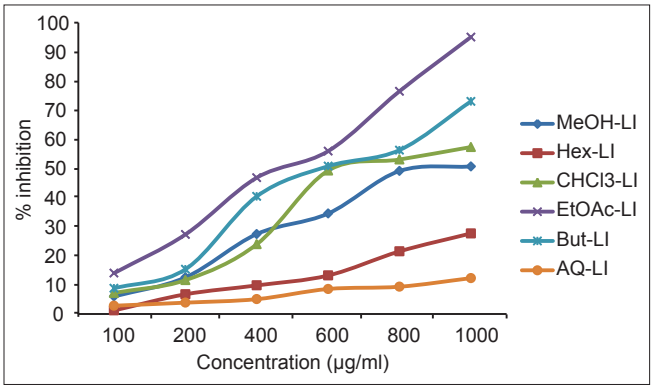

Figure 12: Reducing potential of extract/fractions of *Lawsonia inermis* leaves in comparison to rutin (standard)

DISCUSSION

Oxidative stress contributes to cancer by causing DNA damage.[32] Replication of damaged DNA prior to its repair may lead to permanent changes, which may lead to carcinogenesis.[33,34] First report regarding the mutagenic/genotoxic and carcinogenic nature of 4-NQO came in 1957.[35] Later on, it was reported that 4NQO mutagenicity/

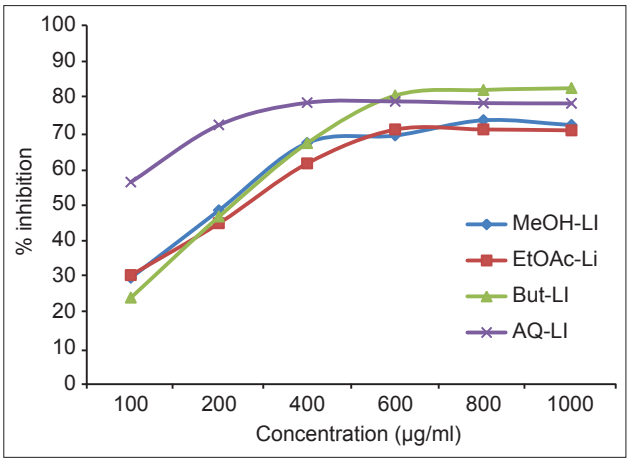

Figure 13: Scavenging effects of extract/fractions of *Lawsonia inermis* leaves on superoxide anion radicals

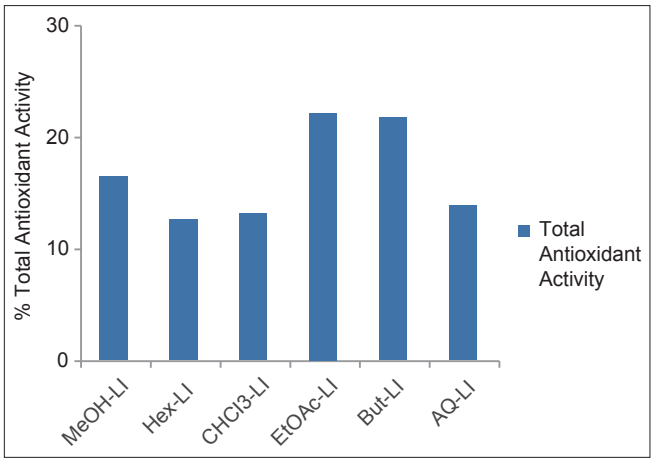

Figure 14: Total antioxidant capacity of extract/fractions of *Lawsonia inermis* leaves

genotoxicity is because of the of its nitro group reduction.[36] The 4-hydroxyaminoquinoline 1-oxide (4HAQO) is produced as result of the four electron reduction of 4-NQO. Further metabolism to selyl-4HAQO (electrophilic reactant),[37] which covalently binds to DNA leading to the formation of quinoline monoadducts: 3-(deoxyadenosin-N6-yl)-4AQO and N4-(guanosin-7-yl-4AQO).[38] 4NQO is known to cause oxidative DNA damage.[39-41] In the present study, it was assessed that EtOAc-LI and MeOH-LI modulated the induction factor induced by 4NQO from 8.85 to 4.42 (52.76%) and 8.85 to 4.81 (51.44%), respectively, in SOS chromotest. Huang *et al*.,[42] reported that Flos Inulae, the dried flower of *Inula japonica* suppressed 4-NQO-induced mutagenicity in Ames assay. They also reported that major phenolic constituents viz., chlorogenic acid, rutin, quercetin, luteolin and kaempferol were responsible for the antimutagenic activity of extracts. Kang *et al*.,[43] reported that aqueous extract of guava twigs inhibited the mutagenicity induced by 4-NQO in *Salmonella typhimurium* and its HPLC analysis showed that the major phenolic constituents present in the extract were gallic acid, ferulic acid and myricetin. These compounds may contributes towards antimutagenic activity of extracts. Tanaka *et al*.,[44] reported that phenolic acids viz., caffeic acid, ellagic acid, chlorogenic acid and ferulic acid inhibited the carcinogenesis of tongue induced by 4-NQO in rats. Nitrofurantoin is a broad-spectrum bactericidal agent with complex action. It is reduced by bacteria to reactive substances that alter or inactivate cell wall synthesis, bacterial ribosomal proteins, DNA, RNA functions pyruvate metabolism and other macromolecules within the cell.[45,46] MeOH-LI modulated the induction factor induced by NF from 4.29 to 2.02 (68.50%) in SOS chromotest. It was found that EtOAc-LI is highly effective against NF-induced genotoxicity showing percent inhibition of 78.42. Bhouri *et al*.,[47] studied antigenotoxic potential of kaempferol 3-O-b-isorhamninoside and rhamnocitrin 3-O-b-isorhamninoside isolated from the leaves of *Rhamnus alaternus* L. Both compounds were found to be good inhibitor of nitrofurantoin induced genotoxicity. Several herbal and medicinal plants possess a potential to protect DNA from electrophilic attack of genotoxins. In the present study, MeoH-LI and EtOAc-LI provided 96.50% and 97.60% protection to pBR322 plasmid DNA, respectively, against damaging effect of hydrogen peroxide via hydroxyl radical generation in Fenton's reagent. The results suggest that these extracts demonstrated hydroxyl radical scavenging that prevented oxidative DNA damage. As there is no specific enzyme that can provides defense in human body against these radicals, so the search for such plant extract/phytochemicals with excellent hydroxyl radical scavenging ability would be useful for treating certain ailments resulting from oxidative damage.[48] Plant extracts can modulate genotoxicity either by acting as desmutagens or bio-antimutagens.[49,50] MeoH-LI and EtOAc-LI exhibited very high antioxidant activity in ABTS assay (98.36 and 99.80%), DPPH assay (86.83 and 91.50%), superoxide anion radical scavenging assay (72.19 and 70.71%) and TAC assay (20.39 and 27.50%). However, EtOAc-Li showed excellent reducing power ability and NO scavenging activity of 95.03% and 54.87% as compared to MeOH-LI (50.63% and 16.08%) respectively. These extracts were rich in phenolic and flavonoid content. HPLC analysis showed that EtOAc-LI and MeOH-LI contain significant quantity of chlorogenic acid (1.738 and 2.203%), Ellagic acid (5.929 and 1.741%), kaempferol (3.985and 0.758%), gallic acid (0.724 and 0.237%) and catechin (1.566 and 0.701%). Previous reports in literature showed that ellagic acid possess antioxidant and antimutagenic activity.[51-56] Kaempferol possessed excellent antioxidant activity in different *in vitro* assays[57-59] and antimutagenic activity in Ames assay.[58] Different studies[60,61] demonstrated that chlorogenic acid has potential to suppress genotoxicity induced by N-methyl-N-nitro-N-nitrosoguanidine by scavenging electrophilic products of the genotoxin and

have good antioxidant activity. Gallic acid exhibited antioxidant activity in electronic paramagnetic resonance method, deoxyribose assay, hydrogen peroxide assay, DPPH assay, chelating power assay and reducing power assay.[62-67] The significant antigenotoxic and antioxidant activity of EtOAc-LI than other extracts may be accredited to the higher content of ellagic acid, kaempferol, chlorogenic acid, catechin and rutin [Figure 2]. But-LI fraction showed moderate inhibition of reactive oxygen species in all antioxidant assays. However, it was found that But-LI showed very good potency to inhibit NF induced genotoxicity showing percent inhibition of 88.68. On the other hand, But-LI showed moderate inhibition of 4-NQO-induced genotoxicity with percent inhibition of 46.37. It has been observed that this fraction provided 100% protection in DNA protection assay. It possessed fair amount of TPC and TFC. Further HPLC analysis showed that it contain moderate amount of catechin, cholorogenic acid, ellagic acid and kaempferol besides other polyphenolics [Figure 2].

Hex-Li, $CHCl_3$-LI and AQ-LI were found to show less antioxidant activity. Hex-LI is found to be least active in all antioxidant assays as well as in SOS chromotest. In most of the assays, it did not achieve 50% inhibition viz., reducing power, superoxide anion radical scavenging, nitric oxide anion scavenging assay and also showed less potency to ameliorate 4-NQO and NF-induced genotoxicity. Phytochemical analysis showed that phenolic compounds were present in very low concentration and flavonoid content is negligible. $CHCl_3$-LI and AQ-LI exhibited good radical scavenging activity than that of Hex-LI but less as compared to MeOH-LI, EtOAc-LI and But-LI. However, both fractions showed good antigenotoxicity against both mutagens viz., 4-NQO and NF. AQ-LI showed significant superoxide anion radical scavenging activity (IC_{50} 27.56 µg/ml). Plant phenols have the capability to inhibit the production of superoxide anions radicals.[68] These extract/fractions exhibited selective anticancer activity i.e. they were effective against colon cancer cell line. It was observed that $CHCl_3$-LI (IC_{50} 86.77 µg/ml) was highly cytotoxic followed by Hex-LI, MeOH-LI, EtOAC-LI and AQ-LI. HPLC analysis of $CHCl_3$-LI fraction demonstrated that it contains kaempferol, quercetin and ellagic acid as major phytoconstituents which may be responsible for the anticancer activity. Flavonoids found in various fruits and vegetables are reported as antiproliferative agents and induces apoptosis in various cancer cells.[69-71] Sharma *et al*.,[72] reported that kaempferol causes apoptosis in glioblastoma cells through reactive oxygen species production. Kaempferol enhances the anticancer effects of cisplatin via decreasing expression of cMyc and inducing apoptosis in ovarian cancer cells.[73] There are several reports in literature that flavonoids reduce the risk of prostate cancer,[74] colorectal cancer[75] and renal cancer[76] Mertens-Talcott *et al*.,[77] reported that ellagic acid and quercetin showed synergic effect, thus enhanced the anticancer effect in MOLT-4 human leukaemia cell line. Han *et al*.,[53] studied that ellagic acid caused apoptosis in human osteogenic sarcoma (HOS) cell via increased expression of Bax and caspase-3. These extracts are under investigation for their mechanism of action against colon cancer.

CONCLUSIONS

This is the first report showing the potential of *Lawsonia inermis* L. phytoconstituents in protecting DNA as well as anticancer activity against colon cancer. The antioxidant activity of these extracts may partially be responsible for the above activities.

ACKNOWLEDGMENT

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