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In vitro assessment on medicinal properties and chemical composition of *Michelia nilagirica* bark

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ABSTRACT

Objective: To outline the antibacterial, antioxidant, α-glucosidase inhibition and anticancer properties of *Michelia nilagirica* (*M. nilagirica*) bark extract.

Methods: The antibacterial activity of bark extracts against human pathogens was assessed by disc diffusion assay. Phytochemical screening, total phenols, flavonoids content, antioxidant and α-glucosidase inhibition properties of bark extracts were investigated by standard methods. *In vitro* anticancer activity of ethyl acetate extract at various concentrations was observed against HepG2 cells using MTT [3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide] assay. The presence of diverse bioactive constituents in the ethyl acetate extract was identified using FT-IR and GC–MS analysis.

Results: Ethyl acetate extract was found to be the promising agent against human pathogens tested. The ethyl acetate extracts showed the presence of various phytochemicals and comprised the substantial content of phenolics and flavonoids. The ethyl acetate extract showed better antioxidant activities and also revealed remarkable reducing power ability and α-glucosidase inhibition property. The dose dependent assay of extract showed remarkable cancer cell death with IC_{50} value of (303.26 ± 2.30) μg/mL. FTIR and GC–MS results indicated the presence of major bioactive constituents in the ethyl acetate extract of *M. nilagirica* bark.

Conclusions: Revealing the first report on *in vitro* biological properties and chemical composition analysis of *M. nilagirica* bark extract, our study implied that this plant could be of great importance in food and pharmaceutical industries.

1. Introduction

In the present scenario, herbal medicines have attracted enormous attention as significant alternatives to commercial drugs in order to treat or prevent life-threatening diseases. Additionally, the natural products derived medicines are found to be more effective with least side effects as compared to commercial therapeutic drugs [1].

Bacterial infections are the leading cause of mortality and morbidity worldwide and the emergence of antibiotic resistant bacteria has become a major global concern at present. Medicinal plants derived therapies have been proven as a quite promising remedy in the treatment of intractable bacterial infections as a replacement to existing synthetic drugs. Most of the infections are associated with the oxidative stress due to free radicals which represent an essential part of aerobic life and metabolism [2]. An antioxidant agent inhibits or delays the oxidation of substrates despite the lower concentration of antioxidant agents [3]. In fact, the scavenging of reactive oxygen species is one of the possible modes of action of antioxidant agents. The different extracts of the various plant parts are the eminent sources of natural antioxidants. Reactive oxygen species may be the root causative factor responsible for human fatal diseases such as cancer through several ways viz. cell membrane disintegration, membrane protein damage and DNA mutation [4]. Free radical

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scavenging properties of medicinal plants have great relevance to the prevention and therapeutics of cancer as alternatives to synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and tertiary butyhydroquinone, which are known for their toxicity and carcinogenic impact on human health.

In addition to this, hyperglycemia leading to obesity and causing diabetes mellitus (DM) is one of the major concerns worldwide. In the current scenario, insulin therapy for preventing DM has several side effects such as resistance to insulin, brain atrophy, anorexia nervosa etc. Thus, an increasing trend in DM has become a serious threat globally that prompts tremendous effort towards exploring new therapeutic agents in order to overcome this devastating situation. In this regard, the inhibition of α-glucosidase could be a beneficial therapy to stem the progress of DM. From these points of views, at present, the quest of efficacious natural antioxidants, antimicrobials and α-glucosidase inhibitory agents from natural resources has become crucial.

Michelia nilagirica (*M. nilagirica*) belongs to the genus *Magnolia* (Magnoliaceae) and it is a native to tropical and subtropical South and Southeast Asia. The plant is endemic to the Western Ghats of India and Sri Lanka. Trees are upto 15 m tall and leaves are simple, alternate and spiral. Flowers are solitary, axillary, large and white. Traditionally, it is being used in leprosy, fever, colic, post partum protection [5] and in eye disorders [6]. In addition to this, the plant also possesses antipyretic, antiulcer, anti-inflammatory, insecticidal and leishmanicidal activities [5,7–9].

Though the detailed characterization and widespread medicinal applications of other species of the genus *Michelia* have been reported, the investigations pertaining to biological activities of *M. nilagirica* are scanty. In view of this, the current context was directed towards investigating the antibacterial, antioxidant, α-glucosidase inhibition and anticancer potentialities of *M. nilagirica.*

2. Materials and methods

2.1. Plant material

The fresh and disease-free plants of *M. nilagirica* were collected from the deciduous forest of Tirumala Hills in Andhra Pradesh, India. The taxonomical identification of the collected plants was authenticated by Dr. K. Madhava Chetty, Plant Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, India. The voucher specimen numbered (LCH406) was deposited at Loyola College herbarium in Department of Plant Biology and Biotechnology. The whole plant of *M. nilagirica* was sorted, cleaned and air-dried at room temperature for 8–10 days. The bark of the collected plants was removed and ground into fine powder. Powdered samples were collected and stored in air- and water-proof containers protected from direct sunlight and heat until required for extraction process.

2.2. Extracts preparation

The powdered bark (500 g) of *M. nilagirica* was mixed successively for 70–72 h into 1.5 L of organic solvents such as ethyl acetate, hexane and methanol in rotator shaker at 130 rpm. The filtrates were further concentrated to dryness in rota evaporator at 40 °C till free from the solvents. The extracts obtained were stored at 4 °C for further *in vitro* studies.

2.3. In vitro antibacterial assessment

2.3.1. Bacteria of interest

The indicator bacteria used for the antibacterial test include Gram positive [*Staphylococcus epidermis* MTTC 3615, *Staphylococcus aureus* MTCC 96, *Enterococcus faecalis (E. faecalis)* MTCC 439 and *Micrococcus luteus* MTCC 106)] and Gram negative [*Shigella flexneri (S. flexneri*) MTCC 1457, *Yersinia enterocolitica* MTCC 840, *Enterobacter aerogens* MTCC 111 and *Proteus vulgaris* MTCC 1771] cultures.

2.3.2. Disc diffusion assay

The Gram (+) bacterial cultures were grown selectively onto Nutrient broth (pH 7.0), whereas Mueller–Hinton broth (pH 7.0) was used for culturing Gram (−) bacteria. The cultures were incubated at 37 °C for 24 h in a rotator shaker. After a required period of incubation, the bacterial cultures (1.5×10^8 CFU/mL) were swabbed on sterile Mueller Hinton agar plates. Different solvents extracts (25 μL) of bark were transferred to sterile discs (6 mm) and allowed to soak for 10–15 min. The discs were transferred aseptically to the plates seeded with the respective pathogens with the help of ethanol dipped and flamed forceps, and incubated at 37 °C for 24 h. After 24 h, zone of inhibition (mm) formed by different solvent extracts of bark against the indicator pathogenic bacteria was measured. Streptomycin and respective solvents soaked in the disc were used as positive and negative control respectively. The experiments were carried out in triplicate.

2.3.3. Determination of relative percentage inhibition

The relative percentage inhibition (RPI) of the bark extracts with respect to positive controls was calculated as described below:

Relative percentage inhibition = (A − B) × 100/(C − B)

Where, A = Total inhibition area of the test extract (πr^2, r = radius of the zone of inhibition).

B = Total inhibition area of the negative control.

C = Total inhibition area of the positive control.

2.3.4. Minimum inhibitory concentration (MIC) determination

The MICs of bark extracts were determined according to the method of Wikaningtyas and Sukandar [10] with some modifications by a serial dilution technique using 96-well microtiter plates. The extracts (1 mg/mL) were placed into the well and then serial dilutions of the crude extracts in broth medium were prepared in a microtiter plate. Further, the bacterial suspensions (100 μL) were added in the microwells at the concentration of 1.5×10^8 CFU/mL. Microtiter plates were incubated for 24 h at 37 °C and the MICs values were calculated as the lowest concentrations preventing visible growth of bacteria. Streptomycin was used as a positive control.

2.4. Qualitative phytochemical screening

The presence of varied phytoconstituents such as alkaloids, phenols, steroids, glycosides, saponins, flavonoids, tannins and reducing sugars in the ethyl acetate, hexane and methanolic extract of bark was assessed in accordance with Evans [11].

2.5. Estimation of total phenolics and total flavonoids content

Total phenolics content (TPC) in the bark extracts of the plant was estimated according to the methods of Singleton *et al.* [12] with some modifications. The reaction mixture contains 1 mL of solvent extract (1 mg/mL), 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% Na_2CO_3. The samples were incubated at 45 °C for 15 min and the absorbance was read at 765 nm. Blank includes ethanol, instead of extract solution. The calibration curve was prepared using gallic acid as standard at the concentrations of 20–100 μg/mL. The total phenolics content was calculated as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract.

The total flavonoids content (TFC) was calculated according to the methodology of Woisky and Salatino [13] with slight modification. The reaction mixture contains 1 mL (1 mg/mL) of the extract, 1.5 mL methanol and 0.1 mL potassium acetate (1 M). Further, the volume was made up to 5 mL using sterile distilled water and the solution was incubated at room temperature for 30 min. The absorbance of the reaction mixture was read at 415 nm. The calibration curve was prepared by using catechol as the standard at concentrations ranging 20–100 μg/mL in methanol.

2.6. In vitro antioxidant properties

2.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The free radical scavenging activity of bark extracts was estimated using DPPH radical according to the methodology of Shimada *et al.* [14] with slight modification. One millilitre of various concentrations of extracts (100–1 000 μg/mL) dissolved in methanol was mixed with 1.0 mL of 0.1 mmol DPPH solution. The reaction mixture was shaken vigorously and incubated for 30 min in dark. The absorbance was measured at 517 nm against a reagent blank. Percentage DPPH scavenging activity of plant extracts was calculated as:

DPPH scavenging capacity (%) = $[(A_{sample} - A_{blank})/A_{control}] \times 100$.

The concentrations of extracts required for 50% inhibition (IC_{50} values) were calculated using standards linear regression curve.

2.6.2. Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 scavenging assay was estimated according to the method of Ruch *et al.* [15] with some modifications. The bark extracts at different concentrations (100–1 000 μg/mL) prepared in ethanol were added to 0.6 mL of H_2O_2 solution. After 15 min of incubation, the absorbance of the reaction mixture was read at 230 nm. Blank solution includes H_2O_2 solution without any extract. H_2O_2 scavenging activity of plant extracts was calculated as:

Hydrogen peroxide scavenging (%) = $(A_0 - A_1)/A_0 \times 100$

where, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

IC_{50} values of extracts were calculated using standards linear regression curve.

2.6.3. Reducing power assay

The reducing power assay for bark extracts was measured according to a methodology of Oyaizu [16] with slight modifications. The reaction mixture contains 2.5 mL of various concentrations of plant extract (dissolved in methanol), 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v in distilled water). The content was mixed well and incubated in a water bath for 15–20 min at 50 °C. After that, 2.5 mL of trichloroacetic acid (10% w/v in distilled water) was added and the mixture was centrifuged at 1 000 rpm for 10 min. The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL ferric chloride (0.1% w/v in distilled water) solution. The reaction mixture was mixed well and absorbance was read at 700 nm. Blank represents the solution devoid of plant extract.

2.7. α-glucosidase inhibition test

α-glucosidase inhibition property of bark extracts (100–1 000 μg/mL) was evaluated according to the methodology of Dahlqvist [17] with slight modifications. IC_{50} values of extracts were calculated using standards linear regression curve. The study was approved by the Institutional Animal Ethical Committee (833/a/04/CPCSEA), Loyola College.

2.8. Anticancer activity of bark extract

2.8.1. Cell line

The human liver cancer cell line (HepG2) was purchased from the National Centre for Cell Science Pune, India. The cell lines were cultured in Dulbecco Modified Eagle's Medium (DMEM). Culture medium was supplemented with 10% fetal bovine serum, antibiotic and antimycotic solution in conditions of 5% CO_2 and 95% air at 37 °C.

2.8.2. In vitro cytotoxicity assay

The ethyl acetate extract showed the magnificent antibacterial, antioxidant and α-glucosidase inhibition properties. Therefore, this extract was chosen for cytotoxicity assay and further characterization. The anticancer activity of ethyl acetate extract of bark on HepG2 cells was determined by the 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay according to the methodology of Mosmann [18] with minor modifications. The cells were harvested (2×10^5 cells/well) and inoculated (100 μL) in 96-well plates. The cells were washed with phosphate buffered saline (PBS) and then inoculated with bark extract (100–1 000 μg/mL). After 72 h of incubation, the medium was aspirated and 25 μL of MTT solution (5 mg/mL in PBS, pH 7.2) was added to each well. The plates were incubated further for 4 h at 37 °C. After required incubation period, 100 μL of DMSO (solublizing reagent) was added to each well and left undisturbed for 1 min. The development of purple colour due to the formation of formazan crystals indicates the presence of viable cells. The absorbance was read at 570 nm using micro plate reader. The concentration of the extract required for a 50% inhibition of cell viability (IC_{50}) was calculated.

2.9. Fourier transform infra-red (FTIR) spectroscopy

Three milligrams of the ethyl acetate extract of bark was mixed with 300 mg of KBr (FTIR grade) and pressed into a

pellet in a hydraulic press by applying 500 kg/m^3 pressure. The pellet was put into the sample holder and FTIR spectra were recorded ranging 4 000–450 cm^{-1} using FTIR spectrometer [Model No.- IRAffinity- 1(SHIMADZU)].

2.10. Gas chromatography-mass spectrometry (GC–MS) analysis

The presence of bioactive compounds in the ethyl acetate extract of bark was analyzed using GC–MS (SHIMADZU QP2010). The GC specifications were as follows: column oven temperature was 70 °C, injector temperature 200 °C, injection mode- Split, Split Ratio- 40, Flow control mode was Linear velocity, Column flow was 1.51 mL/min, Carrier Gas- Helium 99.99% purity. The MS specifications were as follows: Ion source temperature was 200 °C, interface temperature was 240 °C, scan range was 40–1 000 *m/z*, event time- 0.5 s, solvent cut time was 5 min, start time was 5 min, end time was 35 min, and ionization was EI (−70 ev) Ayoola *et al*. [19]. Compounds were identified by their identical GC retention times.

2.11. Statistical analysis

All experiments were carried out in triplicate and results were expressed as Mean ± SD. Statistical analyses were performed using Microsoft Excel 2007 and the IC_{50} values were calculated by simple linear regression curve. Values with $P < 0.05$ were considered statistically significant.

3. Results

3.1. In vitro antibacterial test

The ethyl acetate extract of *M. nilagirica* bark showed broad-spectrum antibacterial activity against Gram (+) and Gram (−) bacteria in a comparison with hexanic and methanolic extract. The ethyl acetate extract of bark showed potent bactericidal activity against *E. faecalis* with maximum zone of inhibition of (14.3 ± 1.3) mm. On the other hand, a minimum zone of inhibition of (10.1 ± 0.4) mm was observed against *S. flexneri* (Table 1). The methanolic extract of bark showed moderate activity against *E. faecalis* and *S. flexneri* with a maximum and minimum zone of inhibition of (10.3 ± 0.6) mm and (8.1 ± 1.1) mm respectively. The hexanic extract of bark was found to be less effective against these pathogens (Table 1). In like manner, the RPI of ethyl acetate extract of bark was found to be the highest against *E. faecalis* with a maximum value of 76.5%. By contrast, the RPI of hexanic extract was estimated to be the least (9.7%) against *Y. enterocolitica*. In accordance to the bactericidal zone of various extracts, the RPI values were found to be affected (Table 1).

3.2. MIC determination

Table 2 shows the MICs values of *M. nilagirica* bark extracts in a comparison with the MICs values of streptomycin against human pathogens. The MICs values of ethyl acetate extract of bark ranged from 31.25 μg/mL to 250 μg/mL, whereas the MICs values ranging from 125 μg/mL to 250 μg/mL correspond to hexanic and methanolic extract of bark. On the other hand, very low concentration of streptomycin 7.81–31.25 μg/mL was found to be promising in order to inhibit the growth of bacteria.

3.3. Qualitative phytochemical screening and quantification of TPC and TFC

The bark extract of *M. nilagirica* was taken into account for the phytochemical screening and estimation of TPC and TFC because of its broad-spectrum antibacterial properties as mentioned earlier. The solvent extracts (ethyl acetate, hexane and methanol) of bark confirmed the presence of various phytoconstituents such as alkaloids, glycosides, phenols, flavonoids and reducing sugars. On the other hand, the qualitative assays showed the lack of saponins, tannins and steroids in the bark extracts (Data not shown).

The present findings showed that the content of total phenolics and total flavonoids differed significantly ($P < 0.05$) among the extracts. The ethyl acetate extract showed substantial

Table 2
MICs values of *M. nilagirica* bark extracts and standard antibiotic against human pathogens.

Bacteria	Bark extracts			Streptomycin
	Ethyl acetate	Hexane	Methanol	
S. epidermis	250.00	250	125	15.62
S. aureus	62.50	250	250	7.81
E. faecalis	31.25	125	250	15.62
M. luteus	125.00	125	250	7.81
S. flexneri	125.00	250	125	15.62
Y. enterocolitica	250.00	250	250	31.25
E. aerogens	250.00	250	125	15.62
P. vulgaris	250.00	250	250	31.25

Unit: μg/mL.

Table 1
Antibacterial activity (Zone of inhibition in mm) and RPI of *M. nilagirica* bark extracts.

Bacteria	Antibacterial activity (mm)						Streptomycin (mm)
	Ethyl acetate (mm)	RPI (%)	Hexane (mm)	RPI (%)	Methanol (mm)	RPI (%)	
S. epidermis	11.3 ± 1.3	37.3	10.3 ± 0.6	30.8	8.2 ± 0.6	19.7	18.3 ± 0.4
S. aureus	12.1 ± 1.2	56.2	6.2 ± 0.4	14.0	8.3 ± 1.1	25.0	16.2 ± 0.3
E. faecalis	14.3 ± 1.3	76.5	6.1 ± 0.3	14.0	10.3 ± 0.6	39.0	16.1 ± 0.4
M. luteus	12.3 ± 0.2	56.2	6.2 ± 0.5	14.0	10.1 ± 1.1	39.0	16.4 ± 0.2
S. flexneri	10.1 ± 0.4	50.9	6.1 ± 0.3	18.3	8.1 ± 1.1	32.5	14.1 ± 0.3
Y. enterocolitica	11.2 ± 0.4	47.2	5.3 ± 0.5	9.7	9.3 ± 0.6	31.6	16.1 ± 0.3
E. aerogens	10.3 ± 0.5	50.9	5.1 ± 0.2	12.7	9.2 ± 0.4	41.3	14.3 ± 0.5
P. vulgaris	10.2 ± 0.4	27.6	6.1 ± 0.4	9.9	10.2 ± 0.3	27.7	19.1 ± 0.3

Values are mean of experiments performed in triplicate and data are expressed as Mean ± SD.

amount of TPC with the highest value of (70.24 ± 1.30) mg GAE/g, followed by hexanic (56.35 ± 1.40) mg GAE/g and methanolic extract (50.34 ± 1.30) mg GAE/g. The TFC for ethyl acetate, hexanic and methanolic extract of bark was estimated as (43.24 ± 1.30), (32.14 ± 1.2) and (22.16 ± 1.30) mg CE/g, respectively (Figure not shown).

3.4. In vitro antioxidant activities

The DPPH radical scavenging property of bark extracts is shown in Figure 1A. All the solvent extracts showed increased scavenging effects in a concentration dependent manner (100–1 000 μg/mL). Among the three solvent extracts studied, the ethyl acetate extract at higher dose depicted maximum antioxidant property of 72.12% ± 1.60% whereas DPPH scavenging activities exhibited by hexanic and methanolic extracts were found to be 58.14% ± 1.30% and 56.64% ± 1.50%, respectively. The dose–response curves of the DPPH radical scavenging activities of the bark extracts were compared with that of standard ($P < 0.05$). The IC_{50} values for ethyl acetate, hexane, methanol extract and ascorbic acid (standard) were estimated at (490.14 ± 1.30), (733.18 ± 1.40), (799.7 ± 1.30) and (138.2 ± 1.50) μg/mL, respectively.

Figure 1B shows H_2O_2 scavenging characteristics of three solvent extracts of bark. The ethyl acetate extract showed maximum scavenging property of 62.12% ± 1.70% at the highest concentration (1 000 μg/mL), whereas the hexanic and methanolic extract depicted maximum antioxidant activities of 52.14% ± 1.6% and 51.64% ± 1.5% respectively at 1 000 μg/mL of concentration ($P < 0.05$). The IC_{50} values for ethyl acetate, hexane, methanol extract and ascorbic acid were estimated at (494.3 ± 1.5), (875.48 ± 1.3), (383.32 ± 1.50) and (964.32 ± 1.30) μg/mL, respectively.

The reductive potentialities of bark extracts at different concentrations are shown in Figure 1C. The bark extracts conferred significant reducing power property which was comparable with that of ascorbic acid. The ethyl acetate extract at higher concentration depicted maximum reducing power ability in terms of absorbance (0.72 ± 0.03). The dose–response curve for the reducing power of hexanic and methanolic extract was found to be decreased significantly ($P < 0.05$) after comparison with ethyl acetate extract.

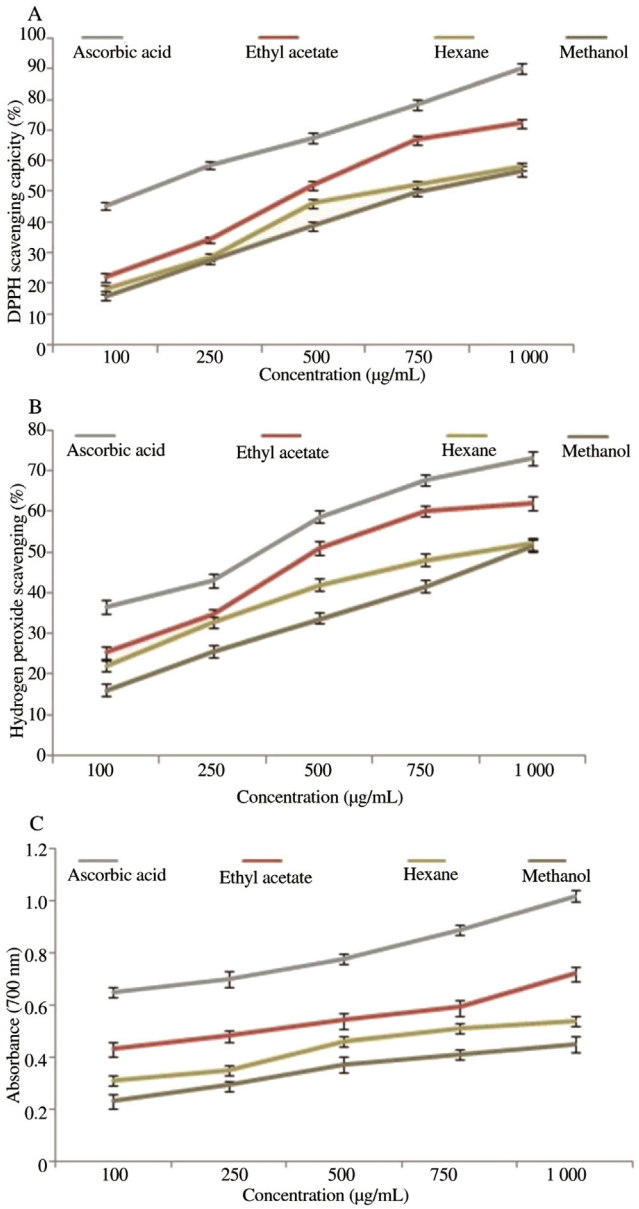

Figure 1. Bark extracts of *M. nilagirica* and ascorbic acid at different concentrations. A: DPPH scavenging activity of bark extracts of *M. nilagirica* and ascorbic acid at different concentrations; B: Hydrogen peroxide scavenging property of plant bark extracts and ascorbic acid at different concentrations; C: Reducing power ability of *M. nilagirica* bark extracts and ascorbic acid at different concentrations. Values are mean of experiments performed in triplicate and data are expressed as mean ± SD.

3.5. α-glucosidase inhibition test

The α-glucosidase inhibiting potentialities of ethyl acetate, hexane and methanolic bark extracts were determined and the results are summarized in Table 3. Ethyl acetate extract showed increased inhibition of α-glucosidase in a concentration dependent manner (100–1 000 μg/mL) with IC_{50} value of (567.6 ± 1.3) μg/mL. The α-glucosidase inhibition properties of bark extracts differed significantly ($P < 0.05$) in the order of ethyl acetate > hexane > methanol. The IC_{50} values for the hexane and methanolic extracts were calculated as (788.8 ± 1.1) and (884.6 ± 1.3) μg/mL, respectively.

Table 3
α-glucosidase inhibition property of bark extracts of *M. nilagirica*.

Extracts (μg/mL)		% inhibition	IC_{50} value (μg/mL)
Ethyl acetate	100	22.12 ± 2.30	567.6 ± 1.3
	250	36.14 ± 1.60	
	500	48.45 ± 1.50	
	750	62.14 ± 1.20	
	1 000	70.34 ± 1.30	
Hexane	100	18.54 ± 1.40	788.8 ± 1.1
	250	29.85 ± 1.30	
	500	38.83 ± 1.30	
	750	48.54 ± 1.40	
	1 000	58.14 ± 1.30	
Methanol	100	16.34 ± 1.30	884.6 ± 1.3
	250	26.64 ± 1.30	
	500	38.84 ± 1.20	
	750	44.14 ± 1.40	
	1 000	53.14 ± 1.30	

Values are mean of experiments performed in triplicate and data are expressed as Mean ± SD.

Table 4
GC–MS analysis for the ethyl acetate extract of *M. nilagirica* bark.

S. No.	Name	Molecular formula	Retention time	Area (%)
1.	Propanoic acid	$C_3H_6O_2$	8.846	0.92
2.	1-Dodecene	$C_{10}H_{21}CH{=}CH_2$	11.847	3.00
3.	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{17}H_{30}OSi$	13.296	6.14
4.	Caryophyllene oxide	$C_{15}H_{24}O$	14.232	0.68
5.	1-Tridecene	$C_{13}H_{26}$	14.306	3.94
6.	1-Naphthalenol	$C_{10}H_8O$	14.982	1.13
7.	1H-Cycloprop(E)azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, (1ar-)1a.alpha,4a.alpha,7.beta,7a.beta,7b.alpha	$C_{15}H_{24}O$	15.561	0.53
8.	Cetene	C_2H_2O	16.535	4.11
9.	Methyl dihydroisosteviol	$C_{21}H_{34}O_3$	16.936	0.49
10.	Lanost-9(11)-en-18-oicacid, 3,20-dihydroxy-23-oxo-, g-lactone, (3b)-(9CI)	$C_{30}H_{46}O_4$	17.775	0.20
11.	1-Nonadecene	$C_{19}H_{38}$	18.563	5.51
12.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	19.536	3.74
13.	Caryophyllene oxide	$C_{15}H_{24}O$	19.618	5.44
14.	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	20.145	0.95
15.	5-Dodecyne	$C_{12}H_{22}$	20.412	4.91
16.	*cis*-Z-.alpha.-Bisabolene epoxide	$C_{15}H_{24}O$	20.531	9.98
17.	Dihydrocarvyl acetate	$C_{12}H_{20}O_2$	20.836	10.56
18.	2-n-octylfuran	$C_{12}H_{20}O$	21.408	11.14
19.	2,3-dihydro-5-benzofuranacetic acid	$C_{10}H_{10}O_3$	21.683	0.81
20.	Trifluoroacetoxy hexadecane	$C_{18}H_{33}F_3O_2$	22.128	1.29
21.	Diethyl Phthalate	$C_{12}H_{14}O_4$	23.25	0.94
22.	Cyclotetracosane	$C_{24}H_{48}$	23.718	0.33
23.	Vitamin E	$C_{29}H_{50}O_2$	27.499	2.43
24.	gamma.-Sitosterol	$C_{29}H_{50}O$	29.044	3.89
25.	Lanosterol	$C_{30}H_{50}O$	29.520	6.44
26.	N-methyl-1-adamantaneacetamide	$C_{13}H_{21}NO$	29.847	0.84
27.	Lanosterol	$C_{30}H_{50}O$	29.936	5.50
28.	1,4-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	31.533	0.91

3.6. In vitro anticancer activity

The ethyl acetate extract of bark at various concentrations (100–1 000 μg/mL) was able to inhibit the proliferation of HepG2 cells. The cell viability was found to be decreased as the concentration of extracts in the culture was increased. The dose dependent study showed 35.52% ± 2.60% and 80.25% ± 1.60% of cell death at the lowest (100 μg/mL) and the highest (1 000 μg/mL) concentrations of ethyl acetate extract respectively. Further, the IC_{50} value of ethyl acetate extract of bark in terms of cytotoxicity was calculated as 303.26 μg/mL (Figure not shown).

3.7. FTIR spectroscopy

The ethyl acetate extract of bark was analyzed by FTIR spectroscopy in order to identify diverse functional groups present in the bioactive components based on its peak ratio as well as electron transition of compounds. In the spectra of bark extract, a broad and strong absorption peak of 3 425.72 cm^{-1} was observed that represents −OH stretching of acid. Strong peaks at 2 927.10 cm^{-1} and 2 860.56 cm^{-1} are due to C−H stretching. The absorption band at 2 351.33 cm^{-1} is mainly due to the presence of O=C=O stretching. Peaks observed at 1 732.15 cm^{-1} and 1 453.43 cm^{-1} are representative for C=O stretching and variable −CH bending. Strong absorption bands at 1 383.02 cm^{-1}, 1 245.10 cm^{-1} and 1 053.18 cm^{-1} are representative for alkyl halide C−F stretching. Strong absorption bands from 822.68 cm^{-1} to 512.12 cm^{-1} are due to alkyl halide such as C−Cl, C−Br and C−I stretching (Figure not shown).

3.8. GC–MS analysis

Table 4 shows the presence of chemical constituents in the ethyl acetate extract of bark that includes bioactive components with their retention time, molecular formula and area percentage. Results depicted that the extracts were complex mixture of 28 bioactive compounds; many of which were present in trace amounts. On the other hand, 2-n-octylfuran (11.14%), dihydrocarvyl acetate (10.56%), *cis*-Z-α-Bisabolene epoxide (9.98%), lanosterol (6.44%) and phenol, 2,4-bis(1,1-dimethylethyl) (6.14%) were identified as the versatile common compounds present in the bark extract.

4. Discussion

In the current scenario, there is a growing interest in the measurement and applications of natural resources and their respective bioactive products for scientific research and varied industrial purposes. Recently, herbal medicines have attracted much attention as alternative medicines for their tremendous role of health benefit. Keeping these facts in view, the present investigation established a major role of *M. nilagirica* as biotherapeutic agents.

In the present context, bark extracts of *M. nilagirica* depicted a significant role as antibacterial agents by successfully inhibiting the growth of Gram (+) and Gram (−) bacterial pathogens. The present finding was partially supported by Khan *et al.* [5] who observed that leaves and seeds extracts of *Michelia* sp. were broadly active against Gram (+) and Gram (−) bacteria. Furthermore, they hypothesized that octadecadienoic acid,

butanoic acid, oleic acid, camphorsulfonic acid, acetic acid and pimaric acid might be responsible for the bactericidal properties. In another study, Kumar *et al*. [20] revealed a narrow spectrum antibacterial activity of *Michelia* sp. against human pathogens including *S. aureus* and *B. subtilis*. Further, the findings of the present study depicted the lower MICs values of ethyl acetate, hexane and methanolic extracts of *M. nilagirica* bark (31.25–250.00 μg/mL) against Gram (+) and Gram (−) bacterial pathogens. The bark extracts, especially ethyl acetate extract, were found to be potent bactericidal agents based on the low MICs values in order to inhibit the growth of tested pathogens. The results are in complete agreement with the reports of Rios and Recio [21] who mentioned that herbal extract possessing an MIC value equalling or less than 1 000 μg/mL is considered to be active and worthy antimicrobials. Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic compounds are the major secondary metabolites of plants that serve as defense mechanisms. Phytoconstituents, especially phenolic components and alkaloids present in plant extracts, play a significant role in their antibacterial properties [22]. In fact, the present study also showed extract possesses a variety of phytochemicals. Therefore, it may be inferred that the antibacterial activities of bark extracts of *M. nilagirica* are due to the availability of high phenolic constituents as well as different concentrations of alkaloids, glycosides, flavonoids and reducing sugars. The promising antibacterial activities of bark extracts provide a preliminary support for the traditional applications of this plant as a source of bioactive components in order to treat various infections.

The phenolics are the largest known groups of secondary metabolites exhibiting antibacterial and antioxidant activities. The number of site(s) and phenol hydroxyl groups leads to the increased hydroxylation, causing relative toxicity to bacteria [23] and scavenging of free radicals [24]. The results of the current study showed that the TPC differed significantly ($P < 0.05$) among different extracts of *M. nilagirica* bark. The TPC of the extracts was compared with the standard gallic acid and the values were found to be maximum for ethyl acetate extract, followed by hexanic extract, with the methanolic extract showing the minimum TPC. A reasonable amount of TPC was quantified in the various extracts of other plants too [25,26]. In fact, phenolic antioxidants are potent free radical terminator which correlates highly with the free radical scavenging property [27].

Flavonoids are also known to exhibit antioxidant activity and reduce free radicals by quenching, upregulating, or protecting antioxidant defences and chelating radical intermediate compounds [28]. The amount of flavonoid concentration was the highest in ethyl acetate extract of *M. nilagirica* bark, followed by hexanic and methanolic extract. In contrary to our findings, Kumar *et al*. [20] observed high flavonoid content in the methanolic extract of *Michelia* sp. In like manner, Venkatachalam and Muthukrishnan [25] and Ahmed *et al*. [26] also reported the maximum TFC in the ethanolic and methanolic extracts of their respective plants. Naturally occurring plant flavonoids have also been reported to exhibit antimicrobial [29,30] and antioxidant activities [31]. The variation in the biological properties of flavonoids is dependent upon the number and positions of methoxy and phenolic groups [32]. Additionally, the present study showed that the TPC in the bark extracts was higher than the TFC, indicating that most of the flavonoids belong to phenolics.

In the DPPH scavenging assay, all the bark extracts showed antioxidant activity in terms of discolouration in a concentration dependent manner and were significantly different ($P < 0.05$). The present findings were in complete agreement with the reports of Motalleb *et al*. [33] who demonstrated the increased scavenging of the DPPH radical with increasing concentration of the samples as well as standards. Results of the current study revealed that the ethyl acetate extract had stronger antioxidant property, conferring the fact that active phytochemicals of *M. nilagirica* bark are readily dissolved in ethyl acetate. The higher DPPH scavenging property of the ethyl acetate extract presumably indicates the presence of a higher content of protic flavonoids in the ethyl acetate extract than the methanolic and hexanic extracts, facilitating hydrogen atom transfer mechanism [26]. The lower DPPH scavenging property of ethyl acetate extract in a comparison with standard ascorbic acid indicates that the reaction of DPPH with ethyl acetate extract of bark was slower than that of standard. Further, the outcomes of the study totally agree with Genwali *et al*. [34] who demonstrated that the extract containing high TFC showed high radical scavenging activity.

Hydrogen peroxide is a weak oxidizing agent that inactivates enzymes by oxidation of essential thiol (-SH) groups and reacts with Fe^{2+} as well as Cu^{2+} ions to form hydroxyl radical which causes toxic effects [35]. In the present study, bark extracts of *M. nilagirica* depicted the scavenging of H_2O_2 in the order of ethyl acetate > hexane > methanol and results were found significantly different ($P < 0.05$) from the standard. The potent H_2O_2 scavenging activity of ethyl acetate extract of bark may be attributed to the presence of high phenolic content which could donate electrons.

The reducing power ability of extracts serves as an indicator of potent antioxidant property. In the present study, the ethyl acetate extract of bark showed better reducing power activity than that of hexanic and methanolic extract, suggesting the presence of enormous amount of reductants in this extract. The reductants have unique ability to break the free radical chain and donate a hydrogen atom [36]. The presence of reductants in the ethyl acetate extract of bark causes the reduction of the Fe^{3+}/ferricynide complex into the ferrous form.

α-glucosidase plays a major role in the bioconversion of carbohydrates into glucose. In fact, the glucose level in the blood can be controlled and maintained up to normal ranges by inhibiting α-glucosidase [37]. The outcomes of the present study provide a significant step towards the inhibition of α-glucosidase in a concentration dependent manner, the ethyl acetate extract being the most active. The α-glucosidase inhibitory property of ethyl acetate extract may be because of the glycoside content. Glycosides consist of sugars that may be structurally similar to carbohydrate which is a substrate of α-glucosidase [38]. IC_{50} value of ethyl acetate extracts of bark was found to be lower than those of hexanic and methanolic extracts because their bioactive components may have a greater synergistic response towards the inhibition of α-glucosidase. Moreover, the greater α-glucosidase inhibitory characteristic of ethyl acetate extract may be due to the higher phenolics and flavonoids content.

There is a growing interest in the pharmacological role of natural sources on cancer treatments and prevention. The results obtained from the present investigation which unambiguously reports that the HepG2 cell line is sensitive to the bioactive components of ethyl acetate extract of bark in a dose dependent manner. Our observations on toxicity against HepG2 were found

to be in complete agreement with the previous report of Sassa *et al*. [39]. The anti-proliferative effect of ethyl acetate extract against HepG2 may be due to the synergistic effects of the various bioactive constituents such as alkaloids, phenols and flavonoids present in the crude extract, finally reducing the cancer risk factors.

FTIR spectrum was used to assess the stability of chemical constituents and the presence of diverse functional groups in the ethyl acetate extract of bark. The present context reported the presence of different peaks in the extract which was supposed to be obtained through stretching and bending vibrations in the region of infrared radiation. In addition to this, different peaks were obtained due to the shifts in the Fourier transform infrared spectra too.

GC–MS analysis of the ethyl acetate extract of *M. nilagirica* bark showed predominant presence of bioactive compounds in the decreasing order of 2-n-octylfuran > dihydrocarvyl acetate > *cis*-Z-α-bisaboleneepoxide > lanosterol > phenol,2,4-bis(1,1-dimethylethyl). The variation in the concentration of these compounds from previously reported other plant extracts could be due to the seasonal variation, different geographical locations, variation in the extraction procedures and choice of solvent. The presence of these bioactive phytoconstituents could be responsible for the strong antibacterial, antioxidant, α-glucosidase inhibition and anticancer properties of ethyl acetate extract of *M. nilagirica* bark. To the best of our knowledge, the study reveals the first report on the chemical composition analysis of the ethyl acetate extract of *M. nilagirica* bark by GC–MS.

The results obtained in the present context are noteworthy, because of not only the promising antibacterial activity, significant TPC and TFC, strong antioxidant activities, potent α-glucosidase inhibition property and good anticancer activity but also the presence of various bioactive components in the bark extract of *M. nilagirica*. The bark extracts, especially ethyl acetate extract was found to be effective antibacterial agents against human pathogens tested. The TPC and TFC were found to be the highest in the ethyl acetate extract, leading to the highest antioxidant and α-glucosidase inhibition activities. Further, the *in vitro* data obtained suggested the significant role of ethyl acetate bark extract as anticancer agent against HepG2 cells at different levels. GC–MS and FTIR analysis revealed the presence of enormous amount of bioactive metabolites such as 2-n-octylfuran, dihydrocarvyl acetate, *cis*-Z-alpha. bisabolene-epoxide, lanosterol and phenol, 2,4-bis(1,1-dimethylethyl) in the ethyl acetate extract of bark. Further *in vivo* safety evaluation of these bioactive compounds needs to be investigated in experimental rodent models prior to its possible commercialization for public uses in future.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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References

[1] Khusro A, Aarti C, Preetamraj JP, Panicker SG. *In vitro* studies on antibacterial activity of aqueous extracts of spices and vegetables against *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI. *Int J Curr Microbiol Appl Sci* 2013; **2**: 79-88.

[2] Tiwari A. Imbalance in antioxidant defence and human diseases: multiple approach of natural antioxidants therapy. *Curr Sci* 2001; **81**: 1179-87.

[3] Matkowski A. Plant *in vitro* culture for the production of antioxidants. *Biotechnol Adv* 2008; **26**: 548-60.

[4] Liao KL, Yin MC. Individual and antioxidant effects of seven phenolic agents inhuman erythrocyte membrane ghosts and phosphatidylcholine liposome systems: importance of the partition coefficient. *J Agric Food Chem* 2000; **48**: 2266-70.

[5] Khan MR, Kihara M, Omoloso AD. Antimicrobial activity of *Michelia champaca*. *Fitoter* 2002; **73**: 744-8.

[6] Sobhagini N, Soumit KB, Malaya KM. Ethno-medico-botanical survey of Kalahandi district of Orissa. *Indian J Tradit Know* 2004; **3**: 72-9.

[7] Ulla J, Vijaya K, Shantini S. Sesquiterpene lactones from Michelia champaca. *Phytochem* 1995; **39**: 839-43.

[8] Vimala R, Nagarajan S, Alam M, Susan T, Joy S. Anti-inflammatory and antipyretic activity of *Michelia champaca* Linn., (white variety), *Ixora brachiata* Roxb. and *Rhynchosia cana* (Willd.) D.C. flower extract. *Indian J Exp Biol* 1997; **35**: 1310-4.

[9] Takahashi M, Fuchino H, Satake M, Agatsuma Y, Sekita S. *In vitro* screening of leishmanicidal activity in Myanmar timber extracts. *Biol Pharm Bull* 2004; **27**: 921-95.

[10] Wikaningtyas P, Sukandar EY. The antibacterial activity of selected plants towards resistant bacteria isolated from clinical specimens. *Asian Pac J Trop Biomed* 2016; **6**: 16-9.

[11] Evans WC. *Trease and Evans pharmacognosy*. 14th ed. Singapore: Harcourt Brace; 1997.

[12] Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 1999; **299**: 152-78.

[13] Woisky R, Salatino A. Analysis of Propils: some parameters and procedure for chemical quality control. *J Agri Res* 1998; **37**: 99-105.

[14] Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autioxidation of soybean oil in cyclodextrin emulsion. *J Agric Food Chem* 1992; **40**: 945-8.

[15] Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 1989; **10**: 1003.

[16] Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutr* 1986; **44**: 307-15.

[17] Dahlqvist A. Method for assay of intestinal disaccharidases. *Anal Biochem* 1964; **7**: 18-25.

[18] Mosmann T. Rapid colorimetric assay for cellular grow and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55-63.

[19] Ayoola GA, Lawore FM, Adelowotan T, Aibinu IE, Adenipekun E, Coker HAB, et al. Chemical analysis and antimicrobial activity of the essential oil of *Syzigium aromaticum* (clove). *Afr J Microbiol Res* 2008; **2**: 162-6.

[20] Kumar RV, Satish Kumar S, Shashidhara S, Anitha S, Manjula M. Antioxidant and antimicrobial activities of various extracts of *Michelia champaca Linn* flowers. *World Appl Sci J* 2011; **12**: 413-8.

[21] Rios JL, Recio MC. Medicinal plants and antimicrobial activity. *J Ethnopharmacol* 2005; **100**: 80-4.

[22] Tran HBQ, McRae JM, Lynch F, Palombo EA. Identification and bioactive properties of endophytic fungi isolated from phyllodes of *Acacia* species. *Curr Res Technol Edu Top Appl Microbiol Microb Biotechnol* 2010: 377-82. Available from: http://www.formatex.info/microbiology2/377-382.pdf.

[23] Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev* 1999; **12**: 564-82.

[24] Sawa T, Nakao M, Akaike T, Ono K, Maeda H. Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor-promoter effect of vegetables. *J Agri Food Chem* 1999; **47**: 397-402.

[25] Venkatachalam U, Muthukrishnan S. Free radical scavenging activity of ethanolic extract of *Desmodium gangeticum*. *J Acute Med* 2012; **2**: 36-42.
[26] Ahmed D, Khan MM, Saeed R. Comparative analysis of phenolics, flavonoids, and antioxidant and antibacterial potential of methanolic, hexanic and aqueous extracts from *Adiantum caudatum* leaves. *Antioxidants* 2015; **4**: 394-409.
[27] Cho SY, Ko HC, Ko SY, Hwang JH, Park JG, Kang SH. Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. *Biol Pharm Bull* 2007; **30**: 772-8.
[28] Ndhlala AR, Finnie JF, Van Staden J. *In vitro* antioxidant properties, HIV-1 reverse transcriptase and acetylcholinesterase inhibitory effects of traditional herbal preparations sold in South Africa. *Molecules* 2010; **15**: 6888-904.
[29] Xu HX, Lee SF. Activity of plant flavonoids against antibiotic-resistant bacteria. *Phytother Res* 2001; **15**: 39-43.
[30] Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* 2005; **26**: 343-56.
[31] Das NP, Pereira TA. Effects of flavonoids on thermal autooxidation of palm oil: structure activity relationship. *J Am Oil Chem Soc* 1990; **67**: 255-8.
[32] Wu T, He M, Zang X, Zhou Y, Qiu T, Pan S, et al. A structure-activity relationship study of flavonoids as inhibitors of *E. coli* by membrane interaction effect. *Biochem Biophys Acta* 2013; **1828**: 2751-6.
[33] Motalleb G, Hanachi P, Kua SH, Fauziah O, Asmah R. Evaluation of phenolic content and total antioxidant activity in *Berberis vulgaris* fruit extract. *J Biol Sci* 2005; **5**: 648-53.
[34] Genwali GR, Acharya PP, Rajbhandari M. Isolation of gallic acid and estimation of total phenolic content in some medicinal plants and their antioxidant activity. *Nepal J Sci Technol* 2013; **14**: 95-102.
[35] Miller MJ, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL, Clark DA. Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J Pharm Exp Ther* 1993; **264**: 11-6.
[36] Gordon MH. *The mechanism of the antioxidant action in vitro*. *Food antioxidants*. Netherlands: Springer; 1990, p. 1-18.
[37] Bosenberg LH, Van Zyl DG. The mechanism of action of oral antidiabetic drugs: a review of recent literature. *J Endocrinol* 2008; **13**: 80-8.
[38] Sugiwati S, Setiasi S, Afifah E. Antihyperglycemic activity of the mahkota dewa [*Phaleria macrocarpa* (scheff.) boerl.] leaf extracts as an alpha-glucosidase inhibitor. *Makara Seri Kesehat* 2009; **13**: 74-8.
[39] Sassa S, Sugita O, Galbraith RA, Kappas A. Drug metabolism by the human hepatoma cell HepG2. *Biochem Biophys Res Commun* 1987; **143**: 52-7.