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Unveiling Breast Cancer Chemo preventive Phytochemicals: GC-MS Profiling and Antioxidant potentials of *Indigofera longiracemosa* Boivin ex Baill

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Abstract

High recovery of various phytochemicals, such as alkaloids, terpenoids, fatty acids, and phenylpropanoids, was confirmed by the acetone extraction of 400 g of plant material, which produced 60 g of crude extract (15% w/w efficiency). Strong antioxidant activity was demonstrated by the DPPH radical scavenging assay, which showed concentration-dependent absorbance decline (0.431-0.171) and % RSA increase (53.7-80.3%) at 20-100 µg/ml; linear regression (Y=0.336X+46.89; R^2=0.993) determined IC50=9.25 µg/ml, which was better than the BHT standard (IC50=12 µg/ml; Y=0.384X+45.392; R^2=0.9999). Alpha-linolenic acid (9,12,15-octadecatrienoic acid), phytol, neophytadiene, trans-indigo, 1H-indole-2,3-dione, and alpha-tocopherol-β-D-mannoside, many of which have been shown to have antioxidant, anti-inflammatory, cytotoxic, and anticancer effects relevant to breast cancer via apoptosis induction, proliferation inhibition, and oxidative stress modulation. This phytochemical synergy highlights the extract's multi-target potential against oxidative damage and carcinogenesis, positioning it as a promising chemopreventive candidate.

Key words : Phytochemicals, Antioxidant activity, DPPH Assay, IC50, GC-MS, Chemopreventive.

With an anticipated 19.3 million new cases and 10.0 million cancer-related deaths in 2020, cancer is one of the main causes of death globally[20]. The unchecked proliferation and spread of aberrant cells are a hallmark of cancer, a complicated and multidimensional process[3]. Genetic changes that impair normal cell division and growth are one of the many causes of cancer[18]. While chemotherapy drugs can prolong life expectancy and offer short-term comfort[24]. Several anticancer drugs have adverse side effects[14]. Traditional medicine has employed plant-derived substances for millennia, and many of them have demonstrated promise in the treatment of cancer[4]. Natural substances have long been

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used to cure and prevent a wide range of illnesses[19], serving as the foundation of the conventional healing system[5]. Furthermore, a larger population may be able to obtain cancer therapy more easily thanks to the utilization of plant extracts as a sustainable and affordable substitute for pricey and frequently unavailable pharmaceutical medications[12].

Typically, phytochemicals or physiologically active substances with varying polarity combine to form plant extracts. Separation continues to be a significant obstacle in the identification and classification of physiologically active chemicals[1]. A combination analytical method called gas chromatography-mass spectroscopy (GC-MS) is utilized to identify and ascertain the substances found in a plant sample[22]. GC-MS is crucial for phytochemical analysis and chemotaxonomic research on medicinal plants that have components that are physiologically active[7].

The Fabaceae family includes the small tree or shrub *Indigofera longiracemosa* Boivin ex Baill, which is indigenous to India, Sri Lanka, and Nepal. It has a cylindrical crown and is between two and five meters tall. It can be found in scrublands and dry deciduous woodlands. In rats, the roots have been shown to have antidiabetic benefits[10], neutralize all snake venoms[21], and have antioxidant[15]. Leaves have traditionally been used to treat wounds and skin conditions[17]. Leaves have antibacterial qualities against certain bacterial strains. The antifungal qualities of seeds can affect some fungus strains[9]. However, no studies on I. *longiracemosa* anticancer potential have been conducted. Because of this evidence, I. *longiracemosa* was my choice for this investigation.

This study involved a sequential analysis of the acetonic extract of *Indigofera longiracemosa* Boivin ex Baill (AEIL), starting with qualitative phytochemical screening, followed by evaluation of antioxidant potential using the DPPH radical scavenging assay and concluding with GC-MS analysis to identify specific bioactive compounds.

Plant collection and extraction :

The leaves of *I. longiracemosa* were collected in October 2022 from the Edayur north area of the Malappuram district of Kerala (Latitude = 10.88035, Longitude = 76.09092). In Coimbatore, Tamil Nadu, India, Dr M.U. Sharief, Scientist 'F', Botanical Survey of India, Southern Circle, Coimbatore, verified the validity of the plant. After 400 g of plant material was shade-dried, it was removed using 80% acetone and left to stand at room temperature for 72 hours. At a regulated temperature of 40 to 50 °C and lower pressure, the extract was filtered and concentrated in a rotary evaporator until dry. The 60 g dark brown solid extract was kept in a vacuum desiccator at 4 °C until needed again.

Fig. 1 *Indigofera longiracemosa* Boivin ex Baill

Preliminary examination of phytochemicals :

The extract was examined using common screening techniques to determine whether it contained different phytochemical ingredients[23]. For the detection of terpenoids, phenols, steroids, alkaloids, tannins, flavonoids, glycosides, etc., a standard procedure was employed.

Antioxidant activity by DPPH Assay :

Procedure :

The method suggested by Blois[2] was used to assess the plant extracts' antioxidant activity against DPPH. A DPPH dilution of 1×10^{-4} M was made in methanol. Each sample was separated into aliquots of 1 mL in the methanolic extract (at four distinct concentrations: 0.1, 0.5, 1, and 2 mg/mL; two replicates per sample and concentration), and 2 mL of DPPH methanolic dilution was then added. After 16 minutes at room temperature in the dark, the absorbance of the mixture was measured at 517 nm using a UV spectrophotometer. The blank was made using DPPH diluted with methanol. The antioxidant activity (% inhibition or % scavenging) is calculated using the formula.

$$\textbf{DPPH Scavenging Activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Standard for DPPH :

One of the most widely used antioxidants, butylated hydroxytoluene (BHT), is suitable for use in DPPH. Which can be used as reference standard for comparing antioxidant activity of plant extract.

IC_{50} *Calculation :*

The IC_{50} value for both sample and standard was calculated using the equation: **IC50= (50-b)/m,** where b is the intercept and m is the slope of the linear regression line[17].

Gas chromatography- mass spectroscopy (GC-MS) :

A Shimadzu Nexis GC 2030 system with an AOC 30/20i autosampler managed by GCMS Solutions software was used for gas chromatography–mass spectrometry (GC-MS) analysis. Samples were automatically added to the system by the autosampler, and separation was accomplished on a 30-meter-long SH I-5Sil MS capillary column with an inner diameter of 0.25 mm and a film thickness of 0.25 µm that was appropriate for low-polarity compounds. To maximize peak resolution while reducing analysis time, the oven temperature was set in a stepwise ramp. Other inert gases could also be used, but helium was the carrier gas[8].

The extraction of 400 g of plant material yielded 60 g of extract, resulting in an extraction efficiency of 15% (w/w). Acetone extraction yielded a diverse range of phytochemicals, including alkaloids, terpenoids, fatty acids, diterpenoids, triterpenoids and other compounds, indicating its effectiveness in extracting a broad spectrum of bioactive compounds.

The DPPH radical scavenging activity of the sample and standard (BHT) was calculated at various concentrations (20-100 µg/ml). The absorbance values of the sample decreased with increasing concentration, with values ranging from 0.431 to 0.171. The percentage RSA (%) of the sample increased with increasing concentration, with values ranging from 53.7 to 80.3. The IC_{50} values were calculated using linear regression analysis of the concentration- response data. For the sample, an IC_{50} value of 9.25 µg/ml, indicating its potent antioxidant activity. The results are presented in Table-1 and Fig. 2.

Table-1. DPPH Radical Scavenging Activity of the Sample

Sr.no	Concentration(µg/ml)	Absorbance of the sample	% RSA	IC_{50}
1.	20	0.431	53.7	9.25
2.	40	0.373	59.6	
3.	60	0.326	64.4	
4.	80	0.242	73	
5.	100	0.171	80.3	

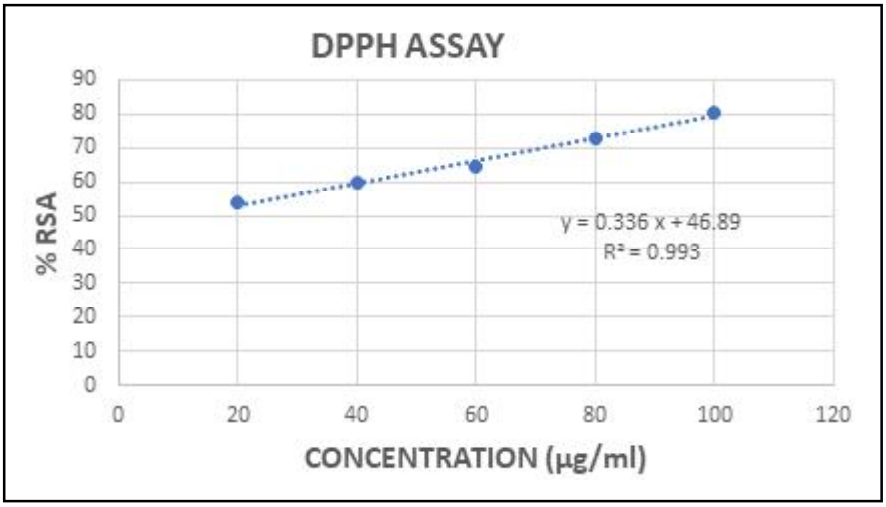


Fig.2. DPPH Radical Scavenging Activity of the sample

In comparison, the standard BHT exhibited absorbance values ranging from 0.485 to 0.202, with % RSA values ranging from 47.6 to 78.2 %. The IC_{50} value of BHT was 12 µg/ml. These results indicate that the sample exhibits antioxidant activity, with a lower IC_{50} value compared to BHT, suggesting higher antioxidant potency. The high R^2 values indicate a strong correlation between concentration and response for both the sample and BHT. The results are presented in Table 2 and Fig. 3.

Table-2. DPPH Radical Scavenging Activity of the standard (BHT)

Sr.no	Concentration(µg/ml)	Absorbance of the BHT	% RSA	IC_{50}
1	20	0.485	47.6	
2	40	0.412	55.5	
3	60	0.342	63.1	12
4	80	0.272	70.6	
5	100	0.202	78.2	

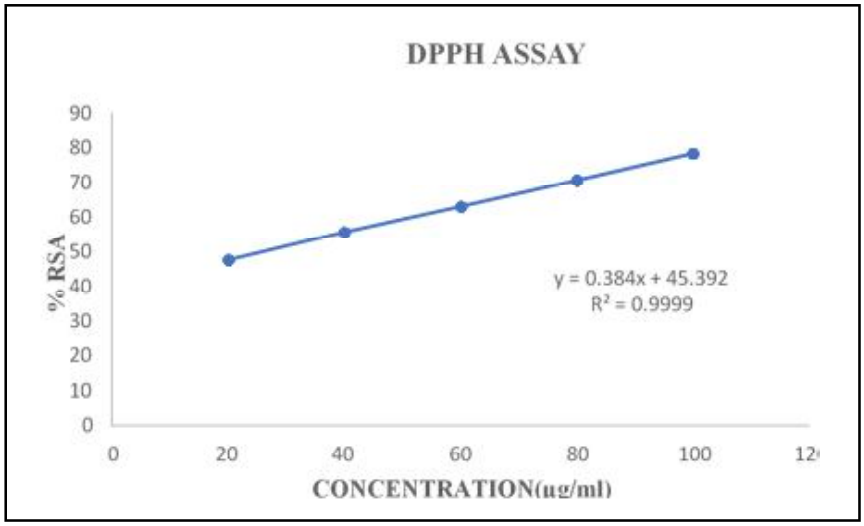


Fig. 3. DPPH Radical Scavenging Activity of the standard (BHT)

The GCMS analysis identified 25 chromatographic peaks in the sample, with retention times, relative peak area percentages, and compound assignments. The data is represented in Fig.4. Those resolved into 17 unique compounds after merging duplicates. Repeated peaks indicate a high abundance of certain constituents.

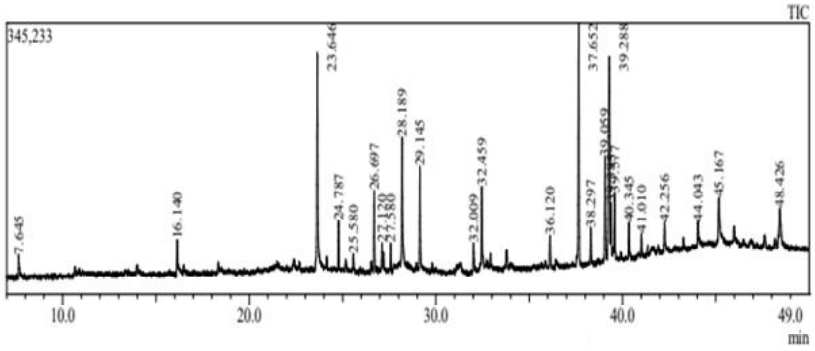


Fig. 4. GCMS Profiling

The bioactive compounds identified in the GCMS exhibit a range of properties relevant to cancer research, including antioxidant, antimicrobial, and potential anticancer activities. Alpha, -tocopherol-. beta. -D-mannoside, a vitamin E derivative, is a potent antioxidant that may help protect normal cells from chemotherapy-induced oxidative stress and potentially alleviate oxidative damage in cancer cells. Trans-indigo, a natural indole pigment, exhibits antimicrobial properties and has been investigated for its anti-leukemic and other anticancer effects, inducing apoptosis in certain cancer cell lines.

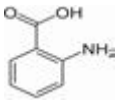
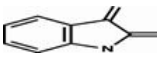
2,3-Diphenylcyclopropyl methyl phenyl sulfoxide isomers, with their unique stereochemistry, are of interest for their potential antioxidant, antimicrobial, and anticancer properties, as sulfoxide-containing compounds are explored for targeting cancer pathways In addition, 9,12,15-Octadecatrienoic acid, an omega-3 fatty acid, is linked to cancer risk reduction and may support treatment efficacy. These compounds collectively suggest the sample may possess antioxidant, antimicrobial, and potential anticancer activities, making them relevant for further investigation in breast cancer and broader cancer research scenarios. The data will be given in Tables-3 and 4.

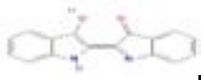
Table-3. GC-MS Peak Data for Compound Identification

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	7.645	98501	1.33	31190	1.42	3.16	MI	Isoamyl ethanoate
2	16.140	155828	2.11	47359	2.15	3.29	MI	Anthranilic acid
3	23.646	869428	11.75	262952	11.95	3.31	MI	1H-Indole-2,3-dione
4	24.787	165425	2.24	61646	2.80	2.68	MI	trans-1,2-Diphenylcyclobutane
5	25.580	60960	0.82	20934	0.95	2.91	MI	2,3-Bis(1-methylallyl) pyrrolidine
6	26.697	283112	3.83	103410	4.70	2.74	MI	Neophytadiene
7	27.120	90704	1.23	34036	1.55	2.66	MI	Anthracene,9-ethenyl-
8	27.580	90944	1.23	36211	1.65	2.51	MI	Neophytadiene
9	28.189	580386	7.85	161955	7.36	3.58	MI	3-Hydroxy-3-methyl-oxindole, Ac derivative
10	29.145	347916	4.70	130035	5.91	2.68	MI	n-Hexadecanoic acid
11	32.009	128501	1.74	36132	1.64	3.56	MI	Phytol
12	32.459	314014	4.25	99634	4.53	3.15	MI	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-

13	36.120	124933	1.69	39723	1.81	3.15	MI	Cyclononasiloxane, octadecamethyl-
14	37.652	901227	12.18	307924	14.00	2.93	MI	Cyclohexane, 1,3,5-triphenyl-
15	38.297	121507	1.64	43976	2.00	2.76	MI	Cyclononasiloxane, octadecamethyl-
16	39.059	403925	5.46	131973	6.00	3.06	MI	(2,3-Diphenylcyc-lopropyl) methyl phenyl sulfoxide, trans-
17	39.288	1157882	15.65	254761	11.58	4.54	MI	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
18	39.385	180876	2.45	66360	3.02	2.73	MI	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
19	39.577	242709	3.28	84435	3.84	2.87	MI	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
20	40.345	147679	2.00	45203	2.06	3.27	MI	Tetracosamethyl-cyclododecasiloxane
21	41.010	90554	1.22	29522	1.34	3.07	MI	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
22	42.256	138189	1.87	37918	1.72	3.64	MI	Tetracosamethyl-cyclododecasiloxane
23	44.043	120504	1.63	30389	1.38	3.97	MI	Tetracosamethyl cyclododecasiloxane
24	45.167	249340	3.37	53279	2.42	4.68	MI	trans-Indigo
25	48.426	331932	4.49	48631	2.21	6.83	MI	Alpha. -Tocopherol-. beta. -D-mannoside

Table-4. GC-MS Analysis of Phytochemical compounds:
Class and Biological Activity

Sl. no	Retention time	Name of the compound	Phytochemical class	Structure of compound	Biological activity
1	7.645	Isoamyl ethanoate	Ester	 $C_7H_{14}O_2$	Antifungal, antibacterial, antiviral, anti-inflammatory & anti-haemolytic.
2	16.140	Anthranilic acid	Aromatic acid	 $C_7H_7NO_2$	Antimicrobial, anti-inflammatory, analgesic, cytotoxic, and antioxidant activities
3	23.646	1H-Indole-2,3-dione	Indole alkaloid	 $C_8H_5NO_2$	Antimicrobial, antidepressant, anticancer, anticonvulsant, and neuroprotective effects,
4	24.787	Trans-1,2-Diphe-nylcyclobutane	Cycloalkene	 $C_{16}H_{16}$	Antimicrobial &antioxidant
5	25.580	2,3-Bis(1-methy lallyl)pyrrolidine	Pyrrolidine alkaloid	 $C_{12}H_{21}N$	Antimicrobial activity & insecticidal
6	26.697	Neophytadiene	Diterpene	$C_{20}H_{38}$	Antimicrobial, antioxidant, anticancer, neuroprotective & cardiovascular.
7	27.120	Anthracene, 9-ethenyl-	Polycyclic aromatic hydrocarbon	 $C_{16}H_{12}$	Antimicrobial, antioxidant, anti-inflammatory, cytotoxicity & anticancer,
8	28.189	3-Hydroxy-3-methyl-oxindole, Ac derivative	Oxindole alkaloid	 $C_{11}H_{11}NO_3$	Antimicrobial, antioxidant, anti-inflammatory & anticancer

9	29.145	n-Hexadecanoic	Fatty acid	 $C_{16}H_{32}O_2$	antioxidant Antimicrobial, & anticancer
10	32.009	Phytol	Diterpene alcohol	$C_{20}H_{40}O$	Antimicrobial, antioxidant, anti-inflammatory, cytotoxicity, anticancer, neuroprotective & cardiovascular,
11	32.459	9,12,15-Octadecatrienoic acid (Z, Z, Z)-	Fatty acid (omega- 3)	 $C_{18}H_{30}O_2$	Antioxidant, anti-inflammatory, cytotoxicity, anticancer, neuroprotective & cardiovascular,
12	36.120	Cyclononasiloxane, octadecamethyl-		 $C_{18}H_{54}O_9Si_9$	Industrial intermediate and cosmetic ingredient
13	37.652	Cyclohexane, 1,3,5-triphenyl-	Cycloalkane derivative	 $C_{24}H_{22}$	Antioxidant & potential anticancer activity
14	39.385	(2,3-Diphenyl-cyclopropyl) methyl phenyl sulfoxide, trans-	Sulfoxide	 $C_{22}H_{20}OS$	Antimicrobial, antioxidant & anticancer
15	40.345	Tetracosamethyl-cyclododeca-siloxane		 $C_{24}H_{72}O_{12}Si_{12}$	No specific bioactivity
16	45.167	Trans-Indigo	Indole alkaloid	 $C_{16}H_{10}N_2O_2$	Colorants, treatment of ulcerative colitis, antimicrobial, antioxidant & potential anticancer activity
17	48.426	Alpha, -Tocopherol-beta. -D-mannoside	Tocopherol	 $C_{35}H_{58}O_{11}$	Antimicrobial, anti-inflammatory, anticancer & neuroprotective antiviral & immunomodulatory

The analysis of the 17 compounds identified in the extract reveals several agents with strong biological activities related to various diseases and health benefits. Compounds such as 9,12,15-Octadecatrienoic acid (Z, Z, Z) exhibit antioxidant, anti inflammatory[13], and proven anticancer properties, which can inhibit breast cancer cell proliferation, induce apoptosis, and potentially manage inflammatory conditions like arthritis[25].

Cyclohexane, 1,3,5 triphenyl, exhibits antioxidant and possible anticancer activity, indicating that it may promote liver health, prevent oxidative stress in diabetes, and inhibit the growth of tumors in breast tissue. 2.3 Diphenylcyclopropyl methyl phenyl sulfoxide exhibits antimicrobial[10], antioxidant, and anticancer properties that can fight infections, target breast cancer cells, and potentially control strains that are resistant to antibiotics[6].

In addition to treating ulcerative colitis and acting as an antioxidant and anticancer agent, trans indigo may have immune-modulatory properties that are beneficial for the treatment of cancer, gut health, and autoimmune conditions like rheumatoid arthritis[11]. The antioxidant, anti-inflammatory, and anticancer properties of alpha tocopherol beta D protect cellular DNA, improve the effectiveness of chemotherapy against breast tumors, lessen the consequences of diabetes, and promote skin health[16].

Acetone proved to be an effective solvent for extracting diverse bioactive compounds from the I. *longiracemosa* plant, yielding 15% (w/w) crude extract rich in alkaloids, terpenoids, triterpenoids, fatty acids, and phenylpropanoids. The high recovery of phenylpropanoids and terpenoids supports the extract's strong antioxidant and potential anticancer properties. DPPH assay results showed concentration-dependent radical scavenging with an IC_{50} of 9.25 µg/ml (R^2 = 0.993), excelling the standard antioxidant BHT (12 µg/ml; R^2 = 0.9999), indicating higher potency. GC-MS analysis revealed 25 peaks representing 17 compounds, including α-linolenic acid, n-hexadecanoic acid, phytol, neophytadiene, indole, oxindole, trans-indigo, and α-tocopherol β-D-mannoside, many of which are known for antioxidant, anti-inflammatory, and anticancer effects. These findings suggest the extract's therapeutic relevance as a natural source of multifunctional bioactive molecules.

Overall, the extraction efficiency, strong DPPH radical scavenging with an IC_{50} lower than BHT, and the phytochemical profile defined by GC-MS suggest that this acetone extract is a rich source of bioactive molecules with significant antioxidant potential and possible relevance to cancer, especially breast cancer. However, antioxidant assays like DPPH represent just an initial in vitro screening step and cannot predict *in vivo* effectiveness or safety on their own. *In vitro* cytotoxicity and mechanism assessments in breast cancer cells, examining apoptosis and cell cycle control, followed by *in vivo* testing in suitable mammary cancer model confirmed the anticancer potential indicated by the current results. Additionally, isolating the most active components through fractionation and bioactivity-guided methods, combined with molecular pathway analysis, will clarify the roles of these phytochemicals and may ultimately lead to new plant-based treatments or lead compounds for breast cancer prevention and therapy.

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