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Phytochemical Investigation and Cytotoxic Activity of Methanolic Extract of Eleusine Coracana

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KEYWORDS

Eleusine coracana, triterpenoid, tetracyclic triterpene, phytosterol, HepG2 cells

ABSTRACT:

In this study, phytochemical investigation and cytotoxic activity of methanolic extracts of Eleusine coracana were performed. Three compounds, stigmasterol, beta-sitosterol and lupeol were isolated from the methanolic extract of Eleusine coracana. These compounds were isolated for the first time from this millet. Methanolic extract of Eleusine coracana was evaluated for the cytotoxic activity at various concentrations against HepG2 liver cancer cells in vitro. The extract showed cytotoxic activity at 250µg/mL. The results of the present proved that Eleusine coracana is active against the cancer due the presence of the active phytochemicals with anticancer properties.

Introduction

Finger millet (*Eleusine coracana* L. Gaertn.) is an ancient crop of significant historical, cultural, and nutritional importance, particularly in Asia and Africa [1-3]. It was first domesticated in Ethiopia and Uganda around 5000 BC, long before it reached India by 3000 BC. The name "finger millet" originates from the shape of its panicles, which resemble several fingers [4]. In terms of nutritional value, *E.coracana* ranks fourth among all millets globally, following sorghum, pearl millet, and foxtail millet. While other millets belong to the Pinaceae tribe, finger millet stands apart as it belongs to the Poaceae family and Chloridoideae subfamily. It is the sole millet belonging to the Chlorideae tribe.

Given its impressive nutritional profile, *E.coracana* is often referred to as a "super cereal." It possesses great potential as a highly nutritious cereal crop that is unfortunately underutilized [5,6]. However, it's important to note that the seed coat of *E.coracana* contains anti-nutritional compounds such as polyphenols, phytates, and tannins, which can hinder the bioavailability of micronutrients. To enhance the bioavailability of the abundant micronutrients present in *E.coracana*, simple processing methods such as soaking, germination, and fermentation, malting, decortication and propping can be employed [7-10].

The antioxidant activity of *E.coracana* is primarily attributed to the presence of polyphenols, phytates, and tannins. These components play a crucial role in promoting overall health and have been explored for their potential in treating various metabolic diseases [11]. Polyphenols identified in *E.coracana* seeds include gallic acid, tannic acid, vanillic acid, ferulic acid, caffeic acid, and chlorogenic acid. High Performance Liquid Chromatography (HPLC) analysis of crude polyphenols extracted from *E.coracana* using HCl-methanol revealed the presence of various compounds. These include

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ferulic acid (32.8%), vanillic acid (3.8%), trans-cinnamic acid (3.6%), p-coumaric acid (4.4%), syringic acid (4.0%), gallic acid (12.6%), proto-catechuic acid (15.3%), p-hydroxybenzoic acid (17.9%), and quercetin (5.6%) [12]. Further analysis of finger millet has revealed the presence of terpenoids, saponins, alkaloids, cardiac glycosides, phenols, balsams, tannins, and steroids [13-16]. The millet also contains plant oxidative enzymes such as lipoxygenase, polyphenol oxidases, ascorbate oxidases, and peroxidases. These enzymes contribute to the millet's high defence mechanism against pests [17].

However, thus so far there is no phytochemistry reported on *E. coracana* to isolate the metabolites. Hence, we aimed to perform the chemical examination of the under-investigated plant *E. coracana* and also to monitor the extract for anticancer activities aginst HepG2 cell line.

Materials and methods:

Collection of *Eleusine coracana*

Eleusine coracana (Finger millet) was collected from Araku Valley, Alluri Seetharama Raju District, Visakhapatnam, Andhra Pradesh on 15th March,2021. It was then identified and authenticated by the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh and voucher specimen was deposited with Coll No.23350 A.U(B.D.H).

Extraction of *Eleusine coracana*

The millet grains (5kg) were shade dried, cleaned and milled to a coarse powder using an electric mill. The required quantity of the whole millet flour (coarsely milled) without debraning and defatting was transferred in to a clean flask and soaked with methanol until the powder was completely submerged. The extract was collected after seven days of maceration. The same process was done for three successive extractions of maceration. During the extraction process, the extracts were filtered using Whattmann Filter No.41 along with 2gm of sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering the extract, the filter paper was made wet by using sodium sulphate and absolute alcohol. The collected filtrate was concentrated at a reduced pressure to obtain a methanolic extract of *E. coracana* (ECM 5gm).

Isolation:

The obtained extract of *E. coracana* was subject to column chromatography to obtain different fractions. 3gms of methanolic extract was adsorbed with 100-200 mesh size of silica gel. The column was packed with 230-400 mesh size silica gel and run with different solvents based on polarity (Hexane-Ethylacetate-Methanol). Fractions are collected at different concentrations. Fraction I (110mg) was obtained as a pale yellow solid, and the observation of the TLC (Hexane: Ethyl acetate, 90:10) showed the presence of one UV active compound with minor impurities, which on treatment with 20% sulfuric acid in methanol turned to dark black. The fraction was re-chromatographed over silica gel (#100-200) using hexane and ethylacetate as an eluent. The sub-fractions 1-12 on evaporation under reduced pressure yielded ECM-1 (10 mg) as a white solid. Fraction II (90mg) was obtained as a yellowish solid. The observation of the TLC (hexane: ethyl acetate, 80:20) showed the presence of two UV active compounds, which on treatment with 20% sulfuric acid in methanol, turned to black. Fraction II was subjected to further column chromatography (#100-200) using hexane in ethyl acetate as eluent. The sub-fractions 1-12 on evaporation under reduced pressure yielded ECM-2 (8 mg) as an amorphous powder. Fraction III (85mg) was obtained as a pale yellow solid, and the observation of the TLC (ethyl acetate: methanol, 75:25) showed the presence of one UV active compound, which on treatment with 20% sulfuric acid in methanol turned to dark black. Fraction III was subjected to further column chromatography (#100-200) using dichloromethane in ethyl acetate as eluent. The sub-fractions 28-29 were concentrated under reduced pressure to obtain ECM-3 (8mg) as a white powder.

Stigmasterol (ECM-1): white solid: melting point:174-176ºC; IR spectrum absorption bands at 3424, 2936, 2867, 1640, 1464 cm^{-1}; molecular formula is $C_{29}H_{48}O$ was established from its EIMS which showed a molecular ion peak at (*m/z*) 412 [M^{+}]. ^{1}HNMR (300 MHz, $CDCl_3$) spectrum demonstrated signals at δ 5.35 (t, *J* = 6.1Hz, 1H), 5.14 (m, 1H), 4.98 (m, 1H), 3.52 (m, 1H), 2.52 - 2.08 (m,5H), 1.98 - 1.92(m, 3H), 1.03 (3H, s), 1.51 (m,1H), 1.52 (m, 2H), 1.32 - 1.40 (m, 3H), 1.18 (m, 2H), 1.14 (m, 2H), 1.01 (s, 3H), 1.01 (s, 3H), 1.02 (m, 1H), 0.96 (m, 1H), 0.91 (d, *J* = 6.2 Hz, 3H), 0.83 (t, *J* = 7.2 Hz, 3H), 0.82 (d, *J* = 6.6 Hz, 3H), 0.80 (d, *J* =

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6.6 Hz, 3H), 0.71 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) shows chemical shifts at δ ppm 140.71 (C-5), 138.2 (C-22), 72.1(C-3), 121.7 (C-6),56.8 (C-14), 56.2 (C-17), 51.1 (C-24), 29.6 (C-25), 42.4 (C-13), 42.6, 40.4, 39.8, 37.5, 36.4, 32.3, 32.1 (C-2), 31.8 (C-7, C-8), 20.9 (C-21), 29.3 (C-16), 18.9 (C-28), 24.4 (C-15), 20.9 (C-21), 21.5 (C-11), 19.3 (C-27), 21.7 (C-19), 12.2 (C-29), 40.6 (C-18) (Table 1).

Beta-sitosterol (ECM-2): white amorphous powder; melting point:134-135 ºC; IR spectrum absorption bands at 3424, 2930, 2852, 1724, 1463, 1378, 1271, 1059, 1023, 963 and 802 cm^{-1} and EIMS showed molecular ion peak at (*m/z*) 414 [M^+] which was consistent with the molecular formula $C_{29}H_{50}O$. ^{1}H NMR ($CDCl_3$, 300 MHz) spectrum indicated peaks at δ 5.36 (1H, d, *J* = 6.4Hz, H-6), 3.53 (1H, m, H-3), 1.01, 0.68 (3H, s, H-19 and H-18), 0.83 (3H, d, *J* = 6.4 Hz, H-21), 0.81 (3H, d, *J* = 6.4 Hz, H-27) and 0.85 (3H, t, *J* = 7.1 Hz, H-26). ^{13}C NMR ($CDCl_3$, 75MHz) showed signals at 37.5 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4),140.9 (C-5),121.9 (C-6), 32.1 (C-7), 32.1 (C-8), 50.3 (C-9), 36.7 (C-10), 21.3 (C-11), 39.9 (C-12), 42.6 (C-13), 56.9 (C-14), 26.3 (C-15), 28.5 (C-16), 56.3 (C-17), 36.3 (C-18), 19.2C-19), 34.2 (C-20), 26.3 (C-21), 36.2 (C-22), 46.1 (C-23), 23.3 (C-24), 12.2 (C-25), 29.4 (C-26), 20.1 (C-27), 19.6 (C-28), 12.0 (C-29) (Table 1).

Lupeol (ECM-3): white powder; melting point:134-135ºC; IR spectrum absorption bands at 3308, 2924, 1465, 1379, 1042 and 880 cm^{-1}; molecular formula $C_{30}H_{50}O$ was assigned based on the molecular ion peak at *m/z* 426 $[M^+]^+$in the ESIMS spectrum. ^{1}HNMR (300 MHz, $CDCl_3$) spectrum demonstrated signals at δ 4.69 and 4.56 (each 1H, s, H-29), 3.16 (1H, dd, H-3), 2.35 and 1.49 (each 1H,m,21A), 1.67 (1H,t, H-15A), 1.71 (3H, s, H-30), 1.61 (2H, d, H-12A, 1A), 1.67 (1H, t, H-13),1.50 (1H, q, H-2B), 1.42 (1H, m, H-22A), 1.41 (2H, m, H-7), 1.50 (1H, q, H-6B), 1.38 (1H, t, H-16A), 1.39 (1H, m, H-21B), 1.25 (1H, q, H-11B), 1.28 (1H, m, H-22B), 1.71 (3H, s, H-26),1.00(3H, s, H-23), 0.94 (3H, s, H-27), 0.92 (1H, t, H-1B), 0.80, 0.79 and 0.73 (each 3H, s, H-25, 28, 29) and 0.66 (1H, d, H-5). ^{13}CNMR **(**75 MHz, $CDCl_3$) spectrum showed signals at δ 150.9 (C-20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.0 (C-18), 48.3 (C-19), 25.1 (C-12), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (C-4), 38.7 (C-1), 38.0 (C-13), 37.1 (C-10), 35.6 (C-16), 34.3 (C-7), 29.1 (C-21), 28.0 (C-23), 27.4 (C-15), 27.4 (C-2), 25.1 (C-12), 28.0 (C-23), 27.4 (C-15), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 18.0 (C-28), 15.9 (C-25), 16.1 (C-26), 15.3 (C-24) and 14.6 (C-27) (Table 1).

Cytotoxity assay: HepG2 cells (procured from ATCC) were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic solution (penicillin-streptomycin) and maintained in the incubator with 5% CO_2 at 37°C. HepG2 cells (3000 cells/well) were seeded in 100 µl DMEM in each well of 96 well plates. After 16 hrs of incubation, cells were treated with test extract of *E.coracana* (ECM) at 1000µg/mL (1 mg/mL), 500µg/mL (0.5 mg/mL), 250µg/mL (0.25 mg/mL) and 125µg/mL (0.125 mg/mL) concentrations in triplicates and were incubated at 37°C in a humidified incubator with 5% CO_2 for 48 hrs.

After 48 hrs, without removing the cell culture media, 100 µl of ice-cold 10% (W/V) Trichloro acetic acid was added to each well, and the plates were incubated at 4°C for 1 hr. Then plates were washed under running water slowly four times, and excess water was removed using paper towels. Next, 100 µl of 0.057% SRB solution was added to each well, plates were incubated at room temperature for 30 min, and then the plates were quickly rinsed four times with 1% (V/V) acetic acid to remove unbound dye.

Further, 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well and shaken for 5 min to solubilize the protein-bound dye. Optical density was measured at 560 nm in a microplate reader.

The percentage of cell inhibition was calculated as:

%cell viability = ((control group value – treated value)/control group value) ×100.

Results and discussion:

The methanol extract of the *E.coracana* was successively performed with methanol. Repeated column chromatography using silica gel of the methanol soluble fractions led to the isolation of three compounds, namely tetracyclic triterpene, phytosterol and triterpenoid (Fig. 1). The structures of the isolated compounds were determined by spectroscopic methods including NMR, IR and Mass spectrometry. To the best our knowledge, compounds 1 - 3 were isolated from *E.coracana* for the first time.

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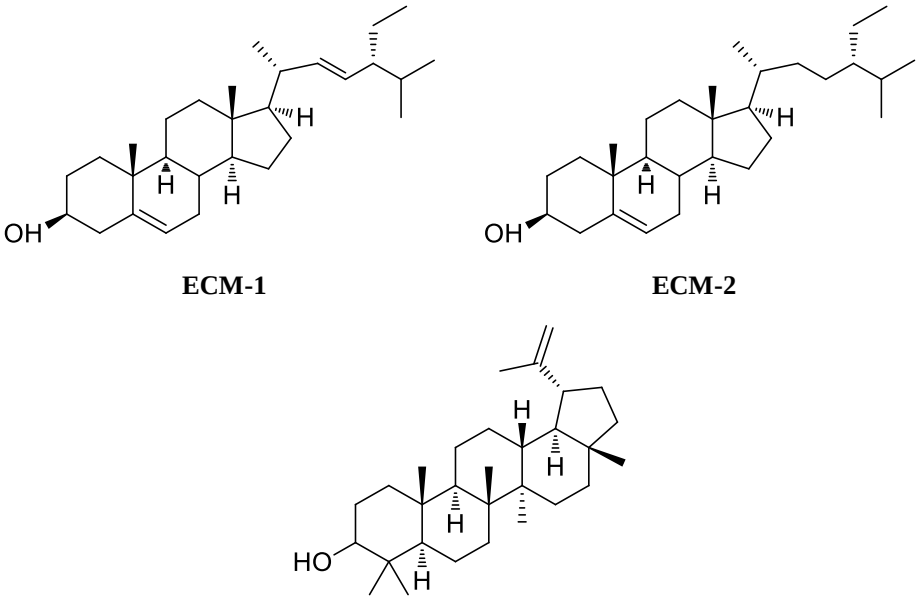

ECM-1 **ECM-2**

ECM-3

Figure 1: structures of compounds 1-3 isolated from *E.coracana*

ECM-1 was obtained as white solid with melting point ranging from 174-176ºC. The IR spectrum absorption bands at 3424, 2936, 2867, 1640, 1464 cm^{-1}. Molecular formula, $C_{29}H_{48}O$ was established from its EIMS which showed a molecular ion peak at (*m/z*) 412 [M^+].The ^{1}HNMR spectra of ECM-1 showed presence of two methyl singlets at δ 0.71 and 1.01, three methyl doublets that appeared at δ 0.80, 0.82 and 0.91 and a methyl triplet at δ 0.83. It also showed protons at δ 4.98, 5.14 and 5.35 suggesting the presence of three protons corresponding to that of a trisubstituted and a disubstituted olefinic bond. The proton corresponding to the H-3 of a sterol moiety was appeared as a triplet of doublet of doublets at δ 3.52. The above spectral data supported the presence of sterol skeleton having a hydroxyl group at C-3 position with two double bonds at C5-C6 and C22-C23 with six methyl groups. Based on IR, Mass, NMR spectral data, the structure of compound was assigned as **stigmasterol**, which was consistent with the reported literature data [18].

ECM-2 was obtained as a white amorphous powder with melting point ranging from 134-135 ºC. The IR spectrum absorption bands at 3424, 2930, 2852, 1724, 1463, 1378, 1271, 1059, 1023, 963 and 802 cm^{-1} and EIMS showed molecular ion peak at (*m/z*) 414 [M^+] which was consistent with the molecular formula $C_{29}H_{50}O$.The presence of six methyl signals that appeared as two methyl singlet's at δ 0.68 and 1.01 (H-19 and H-18), three methyl doublets that appeared at δ 0.81, 0.83, 0.85 (H-27, H-21 and H-26) and a methyl triplet at δ 0.85. This

Table 1: NMR data for compounds 1-3

Carbon No	**ECM-1 NMR data**		**ECM-2 NMR data**		**ECM-3 NMR data**	
	^{1}HNMR(300 MHz,$CDCl_3$) (δ, ppm)	^{1}HNMR (300MHz,$CDCl_3$) (δ, ppm)	^{1}HNMR(400MHz,$CDCl_3$)	^{13}CNMR(75MHz,$CDCl_3$)	^{13}CNMR (75MHz,$CDCl_3$) (δ, ppm)	^{13}CNMR(75MHz,$CDCl_3$) (δ, ppm)
1	--	--	1.61(d, 2H,1a)	38.7	37.5	37.5

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			0.92(t, 1H 1b)			
2	--	--	1.50(q,1H 2b)	27.4	31.9	32.1
3	3.52(m,1H)	3.53(1H,m)	3.16(dd 1H,OH)	79.0	72.0	72.1
4	--	--	--	38.8	42.5	42.3
5	5.35(t,1H,J= 6.1Hz)	--	0.66(d, 1H)	55.3	140.9	140.71
6	--	5.36(d, 1HJ=6.4Hz)	1.50(q, 1H, 6b)	18.3	121.9	121.7
7	--	--	1.41(m, 2H)	34.3	32.1	31.8
8	--	--	--	40.8	32.1	31.8
9	--	--	1.29(m)	50.4	50.3	50.2
10	--	--	--	37.1	36.7	36.4
11	--	--	1.25(q, 1H, 11b)	20.9	21.3	21.5
12	--	--	1.61(d, 2H,12a)	25.1	39.9	39.8
13	--	--	1.67(t, 1H)	38.0	42.6	42.4
14	--	--	--	42.8	56.9	56.8
15	--	--	1.67(t,1H,15a)	27.4	26.3	24.4
16	--	--	1.38(t,1H,16a)	35.6	28.5	23.6
17	--	--	--	42.9	56.3	56.2
18	--	0.68(s,3H)	1.38(m)	48.0	36.3	11.9
19	0.91(d,3H,J= 6.2Hz)	1.01(s)	1.38(m)	48.3	19.2	21.7
20	4.98(m,1H)	--	--	150.9	34.4	40.6
21	5.14(m,1H)	0.83(d,J=6.4H z)	2.35&1.49(m, each 1H, 21a) 1.39(m,1H,21b)	29.1	26.3	20.9
22	--	--	1.42(m, 1H,22a) 1.28(m,1H,22b)	40.0	36.2	138.2
23	--	--	1.00(s,3H)	28.0	46.1	129.2
24	0.83(t,3H,J= 7.2Hz)	--	0.76(s)	15.3	23.3	51.1
25	--	--	0.80(s, 3H)	15.9	12.2	29.6
26	0.82(d,3H,J= 6.6Hz)	0.85(t,J=7.1Hz)	1.71(s, 3H)	16.1	29.4	18.9

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27	0.80(d,3H,J= 6.6Hz)	--	0.94(s, 3H)	14.6	20.1	19.3
28	0.71(s,3H)	0.83(d,J6.5Hz)	0.79(s, 3H)	18.0	19.6	18.9
29	1.01(s,3H)	0.81(t,J=7.5Hz	4.69 & 4.56(s, each 1H) 0.73 (s, 3H)	109.3	12.0	12.2
			1.69(s, 3H)	19.3		

spectrum also showed one olefinic proton at δ 5.36 instead of three in compound. The ^{1}HNMR spectra of compound-2 showed a proton corresponding to the proton connected to the C-3 hydroxyl group which appeared as a triplet of doublet of doublets at δ 3.54. The absence of protons corresponding to the double bond between C22-C23 in compound-2 together with the appearance of mass spectral data supported the presence of sterol having OH group at C-3 position. The presence of a tri substituted double bond at C5-C6 in its structure. Thus, based on these spectral data and in comparison with the literature data, the structure of compound was assigned as **beta-sitosterol** [18].

ECM-3 was obtained as a white powder with melting point ranging from 134-135ºC. The IR spectrum absorption bands at 3308, 2924, 1465, 1379, 1042 and 880 cm^{-1} and molecular formula $C_{30}H_{50}O$ was assigned based on the molecular ion peak at *m/z* 426 $[M^{+}]^{+}$in the ESIMS spectrum. The ^{1}HNMR spectra showed seven tertiary methyl singlets at 0.78, 0.80, 0.86, 0.98, 0.96, 1.06 and 1.68 and one secondary hydroxyl group as doublet of doublets at δ 3.16. It also showed two olefinic protons at δ 4.56 and 4.69 representing the exocyclic double bonds. The ^{13}C NMR of the compound showed 30 signals for the terpenoid which includes a carbon bonded to the hydroxyl group at C-3 position appeared at δ 79.0, while the olefinic carbons of the exocyclic double bond appeared at δ 150.9 and 109.3 ppm. The NMR and mass spectral data suggested that compound-3 is a lupine triterpene having a secondary hydroxyl group at C-3. Based on the above data, structure was thus identified as **lupeol**. The data was compared with the literature and was found to be identical [19].

The cytotoxicity effect of the methanolic extract of *E.coracana* against the HepG2 liver cancer cells were conducted according to the method described in previous paper. Methanolic extract of *E.coracana* showed low percentage of cell viability and high cell cytotoxicity activity at 250µg/mL against HepG2 cells (Fig. 2) (Table 2). These results suggest that cell cytotoxic activity of methanolic extract of *E.coracana* is may be due to the presence of active phytochemicals which have anti-cancer activity. Therefore, these constituents play a major role in exhibiting the cytotoxic activity of methanolic extract of *E.coracana* decreasing the percentage of cell viability in HepG2 cells.

Table 2: Cytotoxic activity of ECM against HepG2 cells.

S.No	**Compound code**	**Concentration (µg/mL)**	**Values of absorbance (OD)**	**Percentage of cell viability**
1	**ECM**	1000 µg	1.417	90.245±19.805
		500 µg	1.899	120.306±14.735
		250 µg	1.148	72.820±2.947
		125 µg	1.4745	93.685±6.202

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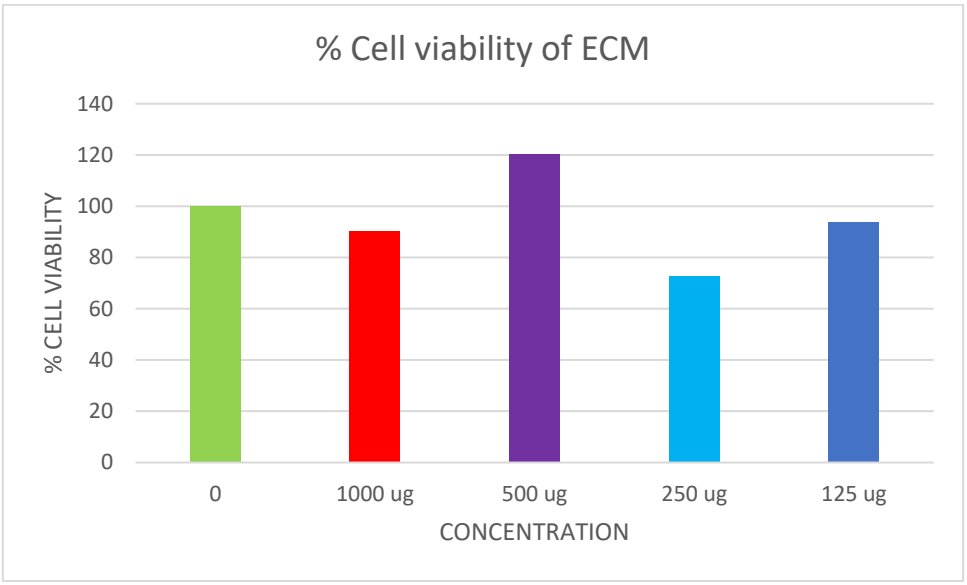

Figure 2: Percentage cell viability of ECM against Hep G2 cells

CONCLUSION

In recent times, pharmaceutical industry is facing challenges to discover novel drugs to improve the quality of the people's health with low toxicity and side effects. Millets promise a novel way of discovering the bioactive compounds as they are rich in polyphenols which have a variety of health benefits such as anticancer, antidiabetic, antimicrobial, antioxidant, hypocholesterolemic effects and also safe guard against diet-related diseases. This study provides valuable insights in to anticancer potentials of *E.coracana* (finger millet). Methanolic extract exhibited significant anticanceer activity as it is rich in polyphenols. The results of the present research work are important to exploit the use of millets, among all other cereals, as a nutraceutical and to promote their use in the prevention of risk of diabetes, cancer and other chronic diseases. Based on the results of the present work, research is further carried on the in vitro and in vivo anticancer activities of the phytochemicals present the millets.

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