Original Research Article

ASSESSMENT OF THE WOUND HEALING PROPERTY OF THE ETHANOLIC SEED EXTRACT OF *Eleusine coracana* Linn USING THE CHORIOALLANTOIC MEMBRANE MODEL

Abstract

Access to a readily available material for wound healing is very important. The wound healing property of *Eleusine coracana* a food source in Nigeria was therefore investigated. The ethanolic crude extract of the seed flour was prepared using maceration and qualitative phytochemical screening was carried out on the crude extract using standard methods. The antimicrobial activity of the *E. coracana* extract was determine using the disc diffusion method against *E. coli, S. aureus, P. aeruginosa, and C. tetani*. The wound healing property assay was carried out using the Chorioallantoin Membrane Model (CAM) at 50mg, 100mg, 150mg and 200mg/ml concentration with the basic Fibroblast Growth Factor (bFGF) as positive control. The crude extract was further fractionated into Hexane, Methanolic and Aqueous fractions and then subjected to amino acid content analysis using the Applied Biosystems PTH Amino Acid Analyzer. The phytochemical screening reveals the presence of alkaloids, steroids, terpenes, flavonoids, carbohydrate, glycoside and protein. *E. coli and S. aureus* were susceptible to the *E. corana* extract at an MIC of 50µg/ml while the *P. aeruginosa* was only susceptible at 200µg/ml and *C. tetani* was not susceptible at all the concentrations used. An increased in percentage angiogenesis was observed at all doses of the extract used indicating good wound healing property. The amino acid profile reveals the presence of glycine, proline that are important in wound healing. The *E. coracana* seeds therefore has wound healing property base on the CAM model.

Key words: Angiogenesis; Wound healing; E. coracana; Antimicrobial; amino acid

INTRODUCTION

Finger Millets (*Eleusine coracana* Linn), is a member of the cereal family and also serve as a good source of food for most of the population of developing countries (Upadhyaya *et al*., 2007). It also has some folklore claims of certain medicinal uses especially in Nigeria (Kareem et al., 2019)

Current estimates indicate that nearly 6 million people suffer from chronic wounds worldwide (Mathieu *et al.*, 2004 and Menke, 2007). Non-healing or chronic wounds result in enormous health care expenditures, with the total cost estimated at more than $3 billion annually (Mathieu *et al.*, 2004 and Menke, 2007). Over the last decades, the search for newer and potent agents from nature (plants, marine environment, fungi and other

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microorganisms) to manage chronic wounds especially, in patients with underlying metabolic disorders has increased immensely (Baranoski and Ayello, 2012; Benbow, 2011).

In order to identify more alternatives for the management of wound this study seeks to verify the wound healing properties of finger millet (*Eleusine coracana* Linn) seeds extract using the Chorio-Allantoic Membrane assay. Since the role of protein has clearly been established in wound healing an assessment of the amino acid content of its seed extracts will also be carried out.

MATERIALS AND METHODS

Collection of plant Material

A two kilograms (2kg) of the plant material finger millet (*Eleusine coracana* Linn) seeds was properly identified and weighed. It was then washed, air-dried washed with distilled water and powdered into flour using a clean dry multiple-pronged electric blender.

Extraction of the seed flour

A two (2) kilograms of the powdered seed flour was soaked with 3L of ethanol in a 10L container for 72 hours at room temperature with intermittent shaking. It was then filtered and the filtrate concentrated with a rotary evaporator at 40°C

Fractionation of the crude extract

The ethanolic crude extract of the *Eleucine coracana* seeds was further fractionated in an open column packed with silica gel (F24) and then eluted with Hexane, Ethyl acetate, Methanol and Water consecutively to yield the Hexane, Ethyl acetate, Methanol and Aqueous fractions respectively.

Qualitative Phytochemical Screening

A test for triterpenoid, glycosides, alkaloids, carbohydrates, saponins, flavonoids, protein, anthraquinones, steroids and tanins was carried out on the crude extract and the respective fractions using standard methods described by Shaikh, & Patil, (2020) and Harbone, (1988).

Amino Acid Analysis/Profiling

The Amino Acid profile in the known sample (Standard), was determined using methods described by Benitez (1989). A 500mg of the standard was defatted using chloroform/methanol (2:1), hydrolyzed, evaporated in a rotary evaporator and a 60µL of the hydrolysate loaded into the Applied Biosystems PTH Amino Acid Analyzer. The hydrolysis was carried out by the use of 7ml of 6N HCl and the sample in a glass ampoule which

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was then sealed and incubated at $105^0C \pm 5^0C$ for 22 hours. The content was then filtered and evaporated to dryness using rotary evaporator. An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids which is used in calculating the amino acid content of the sample. This procedure was repeated using the four fractions Hexane, Ethyl acetate, Methanol and Aqueous fractions.

Percentage Nitrogen Determination

A small amount (115mg) of ground sample was weighed, wrapped in whatman filter paper (No.1) and put in the Kjeldhal digestion flask. Concentrated sulphuric acid (10ml) was added. Catalyst mixture (0.5g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Six pieces of anti-bumping granules were added. The flask was then put in Kjeldhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in standard volumetric flask. Aliquot (10m1) of the diluted solution with 10ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70ml of distillate was collected.

The distillate was then titrated with standardized 0.01 N hydrochloric acid to grey coloured end point. The nitrogen content was then calculated using the formula;

$$\text{Percentage Nitrogen} = \frac{(a-b) \times 0.01 \times 14 \times V \times 100}{W \times C}$$

[a = titre value of the digested sample; b = titre value of blank sample; v = volume after dilution (100ml); W = Weight of dried sample (mg); C = Aliquot of the sample used (5ml); 14 = Nitrogen constant in mg.]

The percentage Nitrogen content was utilized in calculating the amino acid content of each sample using the formula: Concentration (g/100g protein) = NH x Width@ <u>NH</u> x Sstd x C

Where Sstd = N E_{std} x Mol. Weight x μ AA_{std}

%N (Fat Free) =3.20; Volume loaded: 60μL; Dilution = x 5; C=0.009876543

C = (Dilution x 16/Sample Wt (g) x N % x Vol. Loaded) ÷ NH x W (nleucine)

NB: nLeucine is the internal standard

Antimicrobial Activity Assay

A well dried nutrient agar plate was seeded with 1 x 10^6 cfu/ml of test organisms. The excess broth was drained and seeded plate dried. Impregnated discs with samples were placed on the seeded plate at different locations.

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Standard commercial discs were used as control while discs impregnated with sterile distilled water were used as negative control. The plates were incubated at 37°C for 24hrs after which results were read by taking measurement of zones of inhibition. Four organisms were used for the assay namely *E. coli, S. aureus, P. aeruginosa, C. tetani*. The crude extract was prepared in 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml concentrations.

Chorio-allantoin Membrane (CAM) Assay

A Nine day old embryonated eggs were obtained from the Poultry Division, National Veterinary Research Institute (NVRI), Vom. The eggs were candled in the laboratory to ascertain their viability and fitness for use. Viable eggs had their airspace marked out with a pen. The basic Fibroblast Growth Factor (bFGF) gel (commercially obtained from Bharat Biotech Int'l ltd, Hyderabad, India, serial number 092, S8968 5204 5951), was used as positive control while Phosphate Buffered Saline was used as negative control. A 2g of the dried plant extract was appropriately weighed and diluted to prepare final concentrations of 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml. Methyl cellulose discs were prepared and transferred into sterile empty universal discs. The discs were allowed to absorb the controls and samples for over two (2) hours. The eggs were labeled according to the concentrations of extracts to be treated with.

The shell around the marked area of the identified airspace on the embryonated egg was carefully opened using a sterile pair of scissors after an initial incision without tampering with the chorio-allantoin membrane (CAM). Blood vessels were identified and the impregnated discs put along branched vessels according to initial labels. Airspaces were sealed using a transparent adhesive tape and eggs incubated at 37°C in wet chamber for 72hours. Results of angiogenesis were read at 0hour, 24hours, 48hours and 72hours from eggs with live embryos.

RESULTS AND DISCUSSION

The ethanolic extract had flavonoids, tannins, carbohydrates, proteins, cardiac glycosides, steroids and alkaloids. In this extract, saponins, anthraquinones and terpenoids were completely absent as seen on table 1. This corresponds with the work done by Singh and Naithani in 2014, except for alkaloids that were absent and terpenoids present in the extract. This disparity could be due to the species of finger millet, geographical disposition and seed morphology. A similar screening of phytoconstituents was carried out for the fractions resulting from a column chromatography of the ethanolic extract of *Eleusine coracana* Linn. Using the polarity index, the hexane, ethyl acetate, ethanol, methanol and water fractions contains flavonoids, tannins and cardiac glycosides. The most polar ethanol, methanol and water fractions contains alkaloids and proteins. The non-polar hexane and midway polar ethyl acetate contains terpenoids and steroids.

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Except for *Pseudomonas aeruginosa*, all the other organisms were susceptible to 200mg/ml of the extract. On the *Escherichia coli* plate, the zones of inhibition were seen to be dose dependent. The zones of inhibition showed a corresponding increase with those of the extract. The zone of inhibition shown by the standard disc was eminent and those of the graded extract as well, as shown on Table 2. On the *Staphylococcus aureus* plate, the zones of inhibitions were seen to be dose dependent, with corresponding zones of inhibition increasing with increase in strength of the extract.

Table 1: Qualitative Phytochemical Screening data of E. coracana crude extract and fractions

Test	Hexane	EA fraction	EtOH	MeOH	H_2O
Alkaloids	-	-	++	++	+
Saponins	-	-	-	-	-
Tannins	+	+	++	+	+
Flavonoids	+	+	+++	+++	+++
Carbohydrates	-	-	+	+++	++
Proteins	-	-	++	++	-
Terpenoids	+	+	+	-	-
Cardiac glycosides	+	+	+	+	+
Anthraqionones	-	-	-	-	-
Steroids	+	+	+	++	-

Key: - = absence, + = slightly present, ++ = present, +++ = significantly present

A striking observation was that the zone of inhibition for the standard in *S. aureus* is less than that seen in 100mg/ml, 150mg/ml and 200mg/ml of the extract. This is likely so because most microorganisms have different metabolic characteristics. Different microbes that belong to various types of microbial metabolisms have been studied for susceptibility by plant extracts. But among them the group of chemoorganoheterotrophs (organism that obtain carbon, energy, and reducing equivalents needed for several biosynthetic reactions from organic compounds) are the commonly studied for susceptibility testing like *Staphylococcus aureus* (Ohimain *et al*., 2013), as seen on table 2.

The antimicrobial assay for *Pseudomonas aeruginosa*, revealed that the graded doses of the extract showed no zone of inhibition. The standard however showed a clear zone of inhibition. This could be due to

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difference in cell wall characteristics, and this could also contribute to the anti-microbial susceptibility profile of plant extracts. This differs from the work done and reported by Banerjee *et al*. in 2012. In this case, the procured organism could be a resistant strain or even a peculiar one with specific cell wall characteristics.

On the *Clostridium tetani*, the graded doses of the extract showed no inhibition of bacterial growth except for 200mg/ml and the standard. The measurement of the corresponding zones of inhibitions is as captured on table 2. The inhibition at the higher dose and that of the standard on this gram-positive organism could be dose dependent and the metabolic and biochemical characteristics of the organism, especially that gram positive organisms require specificities in handling (Hugo and Russels, 1998).

Table 2: Antimicrobial activity of the crude extract

S/N	MIC (mcg/ml)	ZONES OF INHIBITION (mm)			
		E. coli	*S. aureus*	*P. aeruginosa*	*C. tetani*
1	Solvent	0	0	0	0
2	50	10.4	7.9	0	0
3	100	11.1	8.4	0	0
4	150	11.9	12.1	0	0
5	200	12.1	13.8	0	18.0
6	Standard	15.8	8.1	26.4	18.4

Standasrds: E. coli = ATCC 25922; *S. aureus* = ATCC 25923; *P.aeruginosa* = ATCC 27853

This is to bring a merge between the established physiological processes of wound healing (Flanagan, 2000) and angiogenesis (Carolyn *et al*., 2009) on the blood vessels lining the chorioallantoin membrane (CAM) of the embryonated chick (Rupesh *et al*., 2011). The results could be expressed as percentage angiogenesis, which relates the number of new or dilated blood vessels to the vessels treated with the physiological solution, in this case Phosphate Buffered saline (PBS) expressed in percentage to provide the percentage increase in blood vessels. We could also use an analytical software, where the photographic images of the CAM model are analyzed for quantitative morphometric analysis of the density of blood capillaries in terms of the number of red pixels per unit areas using ImageJ software and AngioQuant software. In both cases, standards of the basic Fibroblast Growth Factor (bFGF) are used and labeled as *in vitro* studies, as described by Barua *et al*. in 2009, Gupta *et a*l. in 2008 and Rupesh *et al*. in 2011. However, the percentage increase in blood vessels was used to express results of angiogenesis.

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The extract was graded, impregnated in commercially purchased methylcellulose discs accordingly then dropped at the junction of a prominent vessel in the airspace of embryonated egg over a period of 48hours.

At 50mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control) and with the PBS (negative control) as described by Rupesh *et al*. in 2011. At 0hour, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hours the vessels were seen to increase in number and dilate, same was done after 48hours and 72hours. The average percentage angiogenesis was 100% for both 50mg extract and extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative control) (See Table 3). Two of the embryos were lost after 48hours of exposure to the extract.

At 100mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control) and with the PBS (negative control) as described by Rupesh *et al*. in 2011. At 0hour, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hours the vessels were seen to increase in number and dilate, same was done after 48hours and 72hours. The average percentage angiogenesis was 75% for 100mg extract and 100% for extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative control). There's a little difference to the information on the work done by Wang *et al*. in 2004, in that, at 50mg we got a100% dilation and newly formed vessels then at increased dosing, we got a reduction. This is likely due to the loss of an embryo or a feedback system from the protest due to an increase in dose (Carolyn *et al*., 2009) as presented in Table 3.

At 150mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control) and with the PBS (negative control) as described by Rupesh *et al*. in 2011. At 0hour, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hours the vessels were seen to increase in number and dilate, same was done after 48hours and 72hours. The average percentage angiogenesis was 91.67% for 150mg extract and 123.077% for extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative control). This was compared to the information on the work done by Wang *et al*. in 2004. Good a thing, there was no loss of an embryo after exposure to the extract. Here, there was a potentiation of extract function by the bFGF (Carolyn *et al*., 2009). This is as shown on table 3.

At 200mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control) and with the PBS (negative control) as described by Rupesh *et al*. in 2011. At 0hour, the prominent blood vessels were located and the extract impregnated methylcellulose discs were

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dropped. The disc was also treated with 10μL bFGF. At 24hours the vessels were seen to increase in number and dilate, same was done after 48hours and 72hours. The average percentage angiogenesis was 100% for 200mg extract and 105.26% extract plus 10μL bFGF, while there was no dilation or new blood vessels on egg treated with PBS (negative control). The glaring presence of the bFGF indicates the potentiated effect of the extract (Carolyn et al., 2009). This is as shown on table 3.

All results for the bFGF alone (positive control) are as shown on table 3. 28.6% at 0hour, while 100% at 24hours, 48hours and 72hours formed the percentage angiogenesis on the blood vessels supplying the embryo.

Table 3: Percentage Angiogenesis of the E. coracana Ethanolic crude extract in the CAM assay

Conc.	**Time**	**Initial Blood Vessels**		**New and Dilated Blood Vessels**		**Percentage Angiogenesis (%)**	
		Ext	Ext+bFGF	Extract	Ext.+bFGF	Extract	Ext.+bFGF
	Neg. Con	0	0	0	0	0	0
50mg	0hour	5	5	5	5	22.7273	29.4118
	24hour	10	5	15	10	45.4545	58.8235
	48hour	15	10	22	15	68.1818	88.2353
	72hour	22	15	22	17	100.00	100.00
100mg	0hour	1	3	1	3	12.5000	21.4286
	24hour	7	8	8	8	87.5000	57.1429
	48hour	8	8	8	8	100.000	57.1429
	72hour	8	14	6	14	75.0000	100.000
150mg	0hour	2	1	2	1	16.6667	6.25000
	24hour	7	6	7	6	58.3333	37.5000
	48hour	12	12	12	12	100.000	75.0000

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	72hour	12	16	11	13	91.6667	123.077
200mg	0hour	3	3	3	3	13.6364	15.0000
	24hour	10	8	10	8	45.4545	40.0000
	48hour	22	20	22	20	100.000	100.000
	72hour	22	20	22	19	100.000	105.2632
bFGF alone	0hour	2		2		28.5714	
	24hour	7		7		100.00	
	48hour	7		7		100.00	
	72hour	7		7		100.00	

Key: Ext = crude extract; Conc.= concentration of extract; Neg.con= Negative control

Amino Acid	Hexane fraction Concentration: g/100g protein	Methanol Fraction Concentration: g/100g protein	Aqueous Fraction Concentration: g/100g protein	Ethanolic Crude Extract Concentration: g/100g protein
Leucine	7.20	4.55	4.00	3.85
Lysine	5.04	2.41	2.70	1.24
Isoleucine	3.30	1.60	1.20	0.95
Phenylalanine	3.55	2.66	1.95	1.06
Norleucine	Internal std	Internal standard	Internal standard	Internal standard
Trytophan	0.84	0.60	0.52	0.44
Valine	3.60	2.22	1.70	0.90
Methionine	1.23	1.04	0.96	0.75
Proline	3.35	2.23	1.83	2.03
Arginine	6.20	4.30	3.01	2.60
Tyrosine	3.44	3.10	2.06	1.03

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Histidine	2.20	1.21	0.86	1.08
Cystine	0.85	0.54	0.36	0.54
Alanine	4.02	2.60	1.82	2.05
Glutamic acid	10.60	5.22	3.63	4.54
Glycine	2.32	3.00	2.10	1.23
Threonine	3.20	1.50	1.05	0.94
Serine	3.35	2.65	2.02	1.51
Aspartic acid	5.98	4.40	3.16	3.10

Table 4: Amino acid content of the three fractions and the Ethanolic extract of *E. coracana* using PTH method

An amino acid analysis was also key looking at the usefulness of proteins in the wound healing cascade. The role of amino acids in wound healing is well established (Albaugh et al., 2017). The synthesis, breakdown, and resynthesis of collagen represents a critical factor in the re-establishment of tissue integrity for wound healing. Many amino acids are very key in collagen synthesis; however, glycine is the most important followed by proline and hydroxyproline (Albaugh et al., 2017).

CONCLUSION

The antimicrobial susceptibility of the seed extract of *E. coracana* showed clear zones of inhibitions against *E. coli, S. aureus, C. tetani* but none against *P. aeruginosa* which signifies its possible importance in wound healing. The angiogenesis by fibroblast mediated activity yielded good percentages using the chorio-allantoin membrane assay clearly indicates that the seed flour extract of *E. coracana* have good wound healing property.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue

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REFERENCES

Albaugh, V. L., Mukherjee, K., & Barbul, A. (2017). Proline precursors and collagen synthesis: biochemical challenges of nutrient supplementation and wound healing. *The Journal of nutrition*, *147*(11), 2011-2017.

Banerjee S., Sanjay K. R., Chethan S. and Malleshi N. G. (2012). Finger millet (*Eleusine coracana*) Polyphenols: Investigation of their Antioxidant Capacity and Antimicrobial Activity. African Journal of Food Science Vol. 6(13): 362-374. DOI: 10.5897/AJFS12.031

Baranoski S. and Ayello E. A. (2012). Wound Dressings: An Evolving Art and Science. *Adv Skin Wound Care*, 25: 87–92.

Barua C., Talukdar A. and Begum S. A. (2009). "Wound Healing Activity of Methanolic Extract of Leaves of *Alternanthera brasiliana* Kuntz using *in vivo* and *in vitro* Model," *Indian Journal of Experimental Biology*, vol. 47, no. 12, pp. 1001–1005.

Benbow M. (2011). Debridement: Wound Bed Preparation. *J Community Nurs*. 25:18–23.

Carolyn A. S., Malcolm W. R. R. and Nicola J. B. (2009). A Critical Analysis of Current *In Vitro* and *In Vivo* Angiogenesis Assays. *Int. J. Exp. Path*., 90; 195 – 221.

Flanagan M. (2000). The Physiology of Wound Healing. Review, *Journal of Woundcare*; 9 (6).

Gupta A., Upadhyay N. K., Sawhney R. C. and Kumar R. (2008). "A Poly-herbal Formulation Accelerates normal and Impaired Diabetic wound healing," *Wound Repair and Regeneration*, 16 (6):784–790.

J.B Harbone. Phytochemical Methods, 3rd Ed. Chapman and Hall: London, 1988, pp. 135-203

Kareem, A. M., Bello, S. O., Etuk, E., Arisegi, S. A., & Umar, M. T. (2019). Evaluation of the hypoglycemic and hypolipidemic effects of aqueous Eleusine coracana seed extract. *International archives of medicine and medical sciences*, *1*(3), 35-41.

Mathieu D., Linke J. C. and Wattel F. (2006). Non-healing Wounds. In: Handbook on Hyperbaric Medicine. *Netherlands: Springer*, 812

Menke N. B., Ward K. R., Witten T. M., Bonchev D. G. and Diegelmann R. F. (2007). Impaired Wound Healing.*Clin Dermatol*. 25: 19–25

Ohimain E. I., Izah S. C. and Jenakumo N. (2013). Physicochemical and Microbial Screening of Palm Oil Mill Effluents for Amylase Production. *Greener Journal of Biological Sciences* 3(8): 314 – 325.

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Rupesh T., Nitika J., Raghvendra P. and Sardul S. S. (2011). Practices in Wound Healing Studies of Plants. Evidence-Based Complementary and Alternative Medicine. *Hadawi Publishing Corporation*; 2(11): 1-17.

Shaikh, J. R., & Patil, M. K. (2020). Qualitative tests for preliminary phytochemical screening: An overview. *International Journal of Chemical Studies*, *8*(2), 603-608.

Singh M. and Naithani M. (2014). Phytochemical Estimation and Antioxidant Activity of Seed Extract of Millets Traditionally Consumed by Common People of Uttrakhand, India. *IJBPAS*; 3(10): 2389-2400

Upadhyaya H. D., Gowda C. L. L. and Reddy V. G. (2007). Morphological Diversity in Finger Millet Germplasm introduced from Southern and Eastern Africa. *J. SAT Agric. Res.* 3 (1) 1–3

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