Modification of a spectrophotometric method to screen hydroxycitric acid producing bacteria

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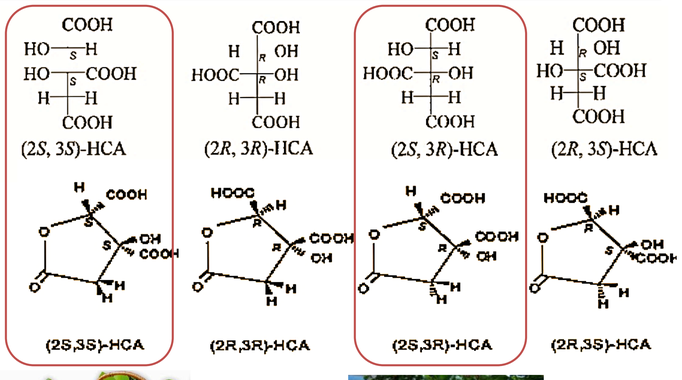
HCA: NATURAL OCCURRENCE, CHEMISTRY AND APPLICATION



Garcinia combogia
Anti-Obesity, Anti-Tumor

- A potent inhibitor of **ATP citrate lyase (EC4.1.3.8)**

Hibiscus subdariffa
Anti-Diabetic

- Inhibits **α- amylase and α-glucosidase**

Jena et al., 2002; Yamda et al., 2007

LIMITATIONS OF PLANT HCA

- Geo-climatically restricted to South Asia.
- Tissue culture and Plant breeding based efforts had limited impact on improving the cultivation of elite HCA producing plant varieties.
- Stereoselective organic synthesis of HCA bioactive isomer is difficult through chemical interventions.

(Hida et al., 2007; Govinder-Soulange et al., 2009; Tembe and Doedhar, 2011)

MICROBES AS AN ALTERNATIVE SOURCE OF HCA

- ***Bacillus megaterium* G45C and *Steptomyces* spp. U121**
- ***Hibiscus* type (*2S,3R*)- HCA; (~2-8mg/L HCA)**

Fermentation technologies and genome shuffling did not prove greatly successful in enhancing bacterial HCA production.

(Hida et al.,2007 ; Yamda et al., 2007

IMPORTANCE OF ISOLATING BACTERIAL ISOLATES CAPABLE TO PRODUCE HIGHER HCA

- Help to understand the prevalence of HCA producing ability within bacterial species.
- Useful to understand metabolic mechanisms.
- Use to identify the genetic engineering targets
- Could be successfully developed into industrially relevant microorganism to optimize large scale bioprocess yielding natural pure biologically active isomers.

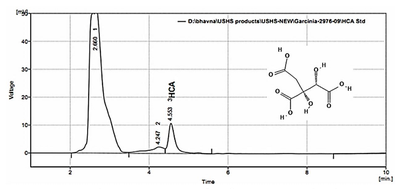
METHODS FOR QUALITATIVE AND QUANTITATIVE ESTIMATION OF HCA

No	Method #		Reference
1	FTIR and HPTLC		
2	IR and ^{1}H NMR	Time consuming for large number of samples	Jayaprakasha et al., 2002; Ravikumar et al., 2017; Soni et al., 2004; Bainto et al., 2018
3	Paper chromatography and TLC		
4	Acid- Base microtitration	Gives total acidity but non specific	Vijay et al., 2009
5	HPLC	Most explored but time consuming	Gogoi et al., 2014; Hida et al., 2005
6	**Spectrophotometric based estimation**	**Convenient but not widely adopted due to instability of coloured complex**	**Antony et al., 1999**

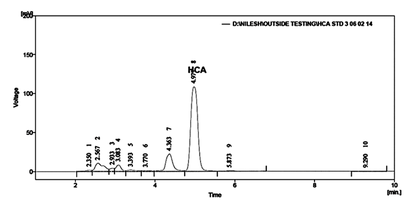
IMPROVISATION OF SPECTROPHOTOMETRIC METHOD TO QUANTIFY HCA

OPTIMIZATION OF HCA STANDARD

- More economic source of Hydroxycitric acid standard
- Containing 350 mg dried *Garcinia* extract



HPLC chromatogram of Ca^{2+}-HCA standard
(VASU research center)



HPLC chromatogram of HCA extract
(VASU research center)
656 ± 67 µg/ml (n=4)

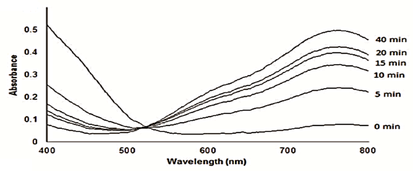
MODIFICATIONS IN ASSAY SYSTEM

Assay system	HCA sample (Prepared in 0.05 N H_2SO_4)	Added component	2.5% sodium metavanadate
AS-1	HCA (1 ml)	-----	+ 0.4 ml
AS-2	HCA (1 ml) +	1 N $NaHCO_3$ (1 ml)	+ 0.4 ml
AS-3	**HCA (1 ml) +**	**1 N NaOH (1 ml)**	**+ 0.4 ml**

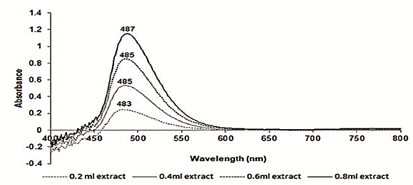
AS- 1: Preparation of metavanadate in **3 N H_2SO_4** (Sinha, 2017) — Could not maintain stability of the complex

AS- 2: Add 1 ml **$NaHCO_3$** to overcome excess acidity

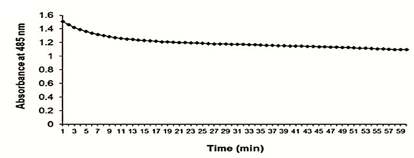
AS-3: Substitute $NaHCO_3$ by **NaOH** to stabilize HCA-metavanadate complex



Instability of HCA-metavanadate complex in presence of excess acidity

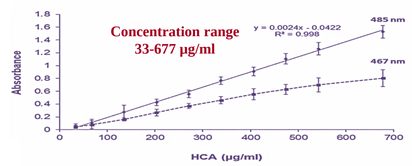


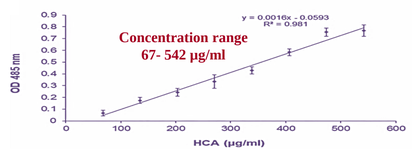
Absorbance spectrum of orange-red complex as obtained using various aliquots with increasing amount of HCA



Time scan of HCA-metavanadate complex over sixty minutes immediately after the addition of the reagent

VALIDATION OF THE PROPOSED METHOD

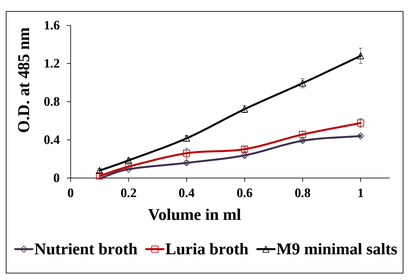




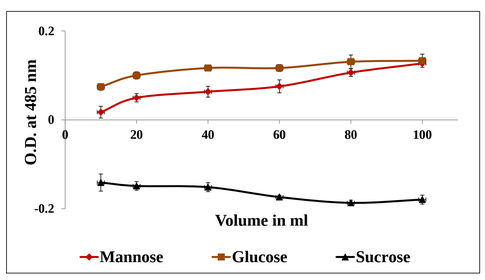
Scaled down assay system (0.24ml total system volume) set up in 96-well plate

SPECIFICITY OF MODIFIED ASSAY SYSTEM WITH RESPECT TO INTERFERENCE FROM GROWTH MEDIA COMPONENTS

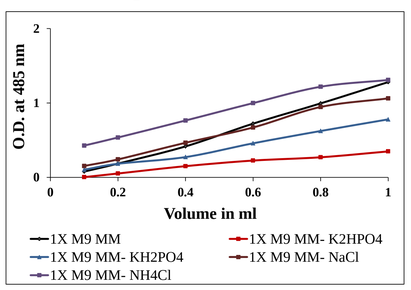
Growth medium interference



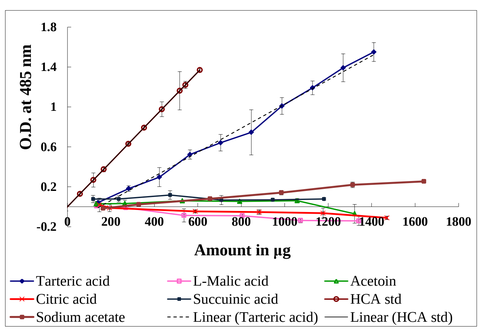
Growth medium interference



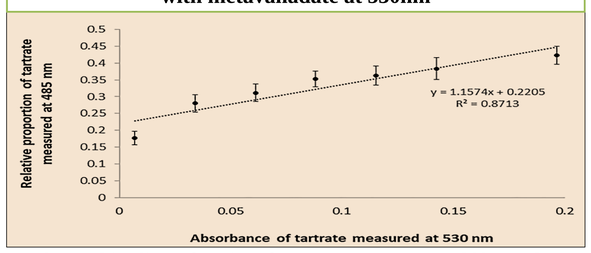
M9 minimal medium (MM) individual component interference



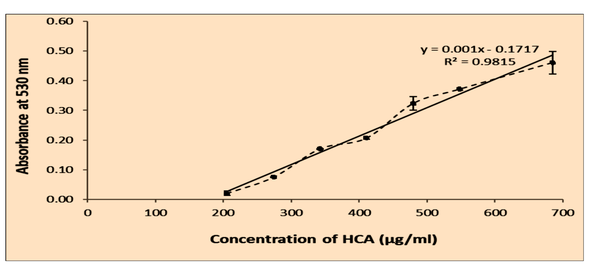
Organic acid interference



Calibration curve of tartaric acid and HCA interference with metavanadate at 530nm

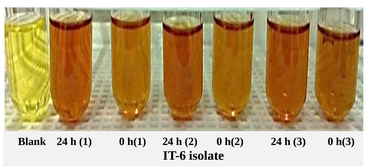


The resulting linear function of y= 1.1574x + 0.2183 could be used to derive unbiased estimate of HCA. (Sales et al., 2001)



Resultant linear curve observed over tested concentration range of 203 – 677 µg/ ml, with equation of y= 0.001x – 0.1717 can be used to determine the proportion of HCA relative to tartrate when measured at 530nm.

Application of proposed method to quantitate HCA from bacterial culture supernatant



Bacterial isolate	HCA measurements (µg/ ml/ OD 600nm)		Tartrate measurements (µg/ ml/ OD 600nm)	
	Spectrophotometric method (with corrections)	HPLC	Spectrophotometric method (with corrections)	HPLC
IT-6	**100.13 ± 35.18** (ns)	**88.28 ±36.90**	**329.98 ±36.88** (ns)	**459.61 ±89.39**

Results are expressed as Mean ± SD of three independent observations; ns represents non significant when compared between spectrophotometric and HPLC measurements.

SUMMARY

Original Assay System

Std volume	0.1 N H_2SO_4	5% $NaVO_3$	20min incubation in dark	λmax 467nm
100 ml	0.2ml			

Modified Assay System

Std volume	0.05N H_2SO_4	2.5% $NaVO_3$	1N NaOH	30min incubation in dark	λmax 485nm
1 ml		0.4ml	1ml		

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