

Biomarkers as a Tool for Validation of Herbs and Spices

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Since ancient times *Piper* species were not only used as medicine but also as spice. It is, therefore, necessary to standardize the *Piper* species on the basis of its biological potentials. In view of this, present work was carried out on various bioefficacies (viz. antimicrobial and antioxidative assay) of *Piper* species and their adulterants to generate biomarkers which can be inexpensive, simple, and rapid means of standardization in heterogenous botanical products. Present investigations suggest that these biomarkers are very effective for genuineness of a drug and simultaneously, also prove that the herbals with high antioxidative value (*P. chaba* RC₅₀ = 0.004 µg/ml) can be safely used for beverages to enhance the quality of the drink with its medicinal potentials.

Herbs and herbal products play important role in curing disease and have potential medicinal value but due to increased industrialization adulteration has become a common feature. Hence, standardization of herbals is a current need. *Piper* species are of high economic and medicinal importance (Nadkarni and Nadkarni, 1954) and several species of *Piper* are ingredients in the formulation of Ayurveda-Chinese-Unani and Compo-System of medicine.

Piper was chemically reviewed by many workers (Parmar *et al.*, 1997; Sengupta and Ray, 1987) but, very little work on their adulteration has been carried out (Madan *et al.*, 1996; Paradkar *et al.*, 2001).

Therefore, *Piper nigrum* Linn. (fruits) and its adulterants [(*Carica papaya* Linn.(seeds), *Lantana camara* Linn.(fruits) and *Embelia ribes* Burm.(fruits)] and *Piper longum* Linn. (fruiting spike) admixed with *P. chaba* Hunter (fruiting spike) and *P. betle* Linn.(leaves) were attempted for their bioefficacies (viz. antimicrobial and antioxidative assay) for generation of various

biomarkers as a tool for standardization and validation.

Biological stability of the herbs with reference to their chemicals relating to medicinal efficacy offers a special advantage in the standardization and quality control of heterogenous botanical products (McLaughlin and Rogers, 1998).

MATERIAL AND METHODS

Plant material. During the course of studies, authenticated samples were procured from different sources: *Piper nigrum* Linn.(Piperaceae) and *Carica papaya* Linn. (Caricaceae) from M/s. Suttind Seeds Pvt. Ltd., Delhi; *P. longum* Linn. (Piperaceae), *P. chaba* Hunter (Piperaceae), *P. betle* Linn.(Piperaceae) and *Embelia ribes* Burm. (Myrsinaceae) from NIA Pharmacy, Jaipur.

Market samples: Various market samples of *P. nigrum* Linn. were procured from M/s. Ajeet Singh Bhag Singh, Jaipur and other *Piper* species were procured from M/s. Chunilal Attar, Jaipur and *Lantana camara* Linn. (Verbenaceae)

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fruits were collected from the Campus of University of Rajasthan, Jaipur, in the months of March - April, 2004.

Antimicrobial assay

Preparation of test extract. Powdered drug (50 g) of all the four selected *Piper* species and their adulterants were successively Soxhlet extracted with pet. ether (60°-80°C), benzene, chloroform, alcohol and water, filtered, concentrated to dryness *in vacuo*. Later, each was dissolved in respective solvents to make 40 mg / 10 disc i.e. 4 mg/disc and used for the studies.

Bacteria. Pure cultures of all test organisms, namely *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* were obtained through the courtesy of SMS Medical College, Jaipur, India, which were maintained on Nutrient Broth medium.

Fungi. The pure cultures of test fungi, namely *Penicillium crysogenum* (5476), *Trichophyton rubrum* (2327), *Candida albicans* (4718) and *Aspergillus niger* (322) were obtained from the IARI, New Delhi, India which were maintained on Potato Dextrose Agar (PDA) medium.

Bactericidal and fungicidal assay. For both, bactericidal and fungicidal assays *in vitro* Disc diffusion method was adopted (Gould and Bowie, 1952) using gentamycin (10 mcg/ml) or mycostatin (100 units/ml) as reference drugs respectively. The inhibition zones in each case were recorded and the activity index (AI) was calculated.

Antioxidative assay

Preparation of test extracts. Powdered drug of selected *Piper* species and their adulterants were milled and refluxed in ethanol for 36 h, filtered and concentrated to dryness *in vacuo*. A portion of ethanolic extract was further successively extracted in pet. ether, benzene, chloroform, alcohol and water, concentrated and stored at minimum temperature, until used.

Preparation of DPPH. 2, 2'-diphenyl-1-picrylhydrazyl (DPPH; C₁₈H₁₂N₅O₆; Hi media)

0.8 mg was dissolved in 10 ml methanol to obtain a concentration of 0.08 mg/ml (Takao *et al.*, 1994) for antioxidative (qualitative and quantitative) assay.

Qualitative and quantitative assay. Each successive extract (10 mg) was dissolved in 10 ml of its suitable solvent to get a concentration of 1 mg/ml and from this, 0.25 µl was taken, applied on silica gel G plates, sprayed with DPPH solution and allowed to stand for 30 min. The change in the colour (from deep- violet to light- yellow) was recorded at 517 nm on a UV spectrophotometer (Varian Cary PCB 150, Water Peltier System). A concentration of 1 mg/ml of ethanolic extract of each test sample was prepared to obtain different concentrations (10²µg to 10⁻³ µg/ ml), out of which 2.5 ml was mixed with DPPH (2.5 ml). The samples were kept in the dark for 15 min at room temperature and the decrease in absorption was measured (at 517 nm) against a blank. The experiment was done in triplicate and the mean was taken for each concentration. Data were processed using EXCEL and concentration that cause 50% reduction in absorbance (RC₅₀) was calculated. The same procedure was also followed for the standards- quercetin and ascorbic acid.

RESULTS AND DISCUSSIONS

Antimicrobial assay

Various sequential extracts of *P. nigrum* and its adulterants (*C. papaya*, *E. ribes* and *L. camara*) and other *Piper* species were screened for various test microbes and their inhibition zones and activity indices were calculated (Tables 1- 2). Benzene extract of *P. nigrum* was active (IZ 7 mm) against *P. aeruginosa* as compared to its adulterants with no activity. On the contrary, the ethanol extract of the adulterants exhibit activity whereas *P. nigrum* was with no activity (Table 1). Among other *Piper* species, most of the extracts were active against one or another fungi. Similarly pet. ether extracts demonstrated greater activity against test fungi for eg. against *C. albicans*, (*P. chaba*,

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IZ 22 mm > *P. betle*, IZ 18 mm > *P. longum*, with no activity) (Table 2). Thus, these “bioefficacy markers” will safely justify the genuineness of the drug and will not only be used as ‘marker’ for Standardization but also provide safety in its quality control.

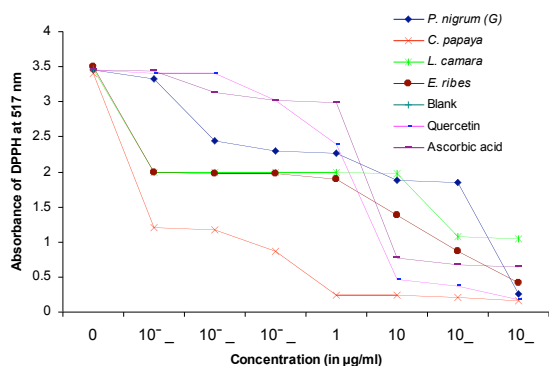


FIGURE 1. Effect of the concentration of ethanolic extract of *P. nigrum* and its adulterants with reference to standard quercetin and ascorbic acid.

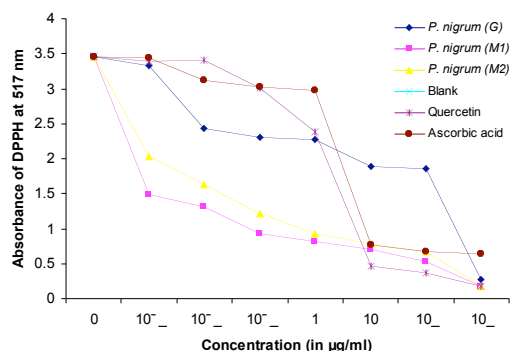


FIGURE 2. Effect of the concentration of ethanolic extract of *P. nigrum* (genuine and market sample 1 and 2) with reference to standard quercetin and ascorbic acid.

Qualitative and quantitative antioxidative assay

The qualitative assay genuine and adulterants of *P. nigrum* indicated the presence of antioxidant component(s) in variable manner (*C. papaya* > *E. ribes* > *L. camara* > *P. nigrum*) like other *Piper* species. In a quantitative DPPH assay, genuine sample *P. nigrum* showed some antioxidant properties ($RC_{50} = 115 \mu\text{g/ml}$; for quercetin $RC_{50} = 4 \mu\text{g/ml}$ and ascorbic acid $RC_{50} = 8 \mu\text{g/ml}$), while

comparing its adulterants *C. papaya* ($RC_{50} = 0.001 \mu\text{g/ml}$), > *E. ribes* ($RC_{50} = 8.5 \mu\text{g/ml}$) and > *L. camara* ($RC_{50} = 25 \mu\text{g/ml}$) with greater antioxidant properties (Figure 1). The high antioxidative RC_{50} value of *C. papaya* as compared to *P. nigrum* market samples I ($RC_{50} = 0.001 \mu\text{g/ml}$) and II ($RC_{50} = 6 \mu\text{g/ml}$) was indicative of the adulteration (Figure 2).

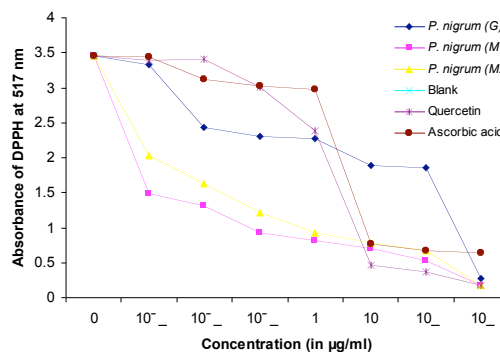


FIGURE 3. Effect of the concentration of ethanolic extract of *P. longum* and *P. chaba* with reference to standard quercetin and ascorbic acid.

Other *Piper* species also exhibited antioxidant potentials (*P. chaba* $RC_{50} = 0.004 \mu\text{g/ml}$ > *P. longum* $RC_{50} = 0.1 \mu\text{g/ml}$ > *P. betle* $RC_{50} = 0.6 \mu\text{g/ml}$) (Figure 3, 4) as compared to the standards (quercetin $RC_{50} = 4 \mu\text{g/ml}$ and ascorbic acid $RC_{50} = 8 \mu\text{g/ml}$).

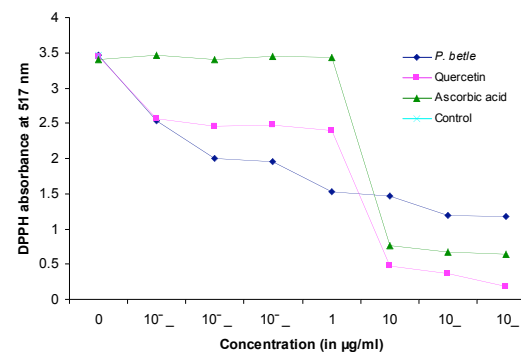


FIGURE 4. Effect of the concentration of ethanolic extract of *P. betle* with reference to standard quercetin and ascorbic acid.

From the results it is evident that biological

profiling in terms of anti-microbial and antioxidative efficacies of the drug plants using selected bacteria and /or fungi following the established simple method(s) can be used as an "effective bioefficacy marker" to differentiate the genuine and the adulterant and/or the resultant mixture which will help the society for establishing the effective biomarkers to determine the nature and degree of adulterants. These simple bioefficacy markers can become increasingly devoted to the identification of bioactive botanical products and their standardization. The antioxidant activity will open the door for herbal drugs as potential beverages which can enhance the quality of our food / drinks and be healthful drinks.

ACKNOWLEDGEMENT

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REFERENCES

- Gould, J. C., and J. H. Bowie. 1952. The determination of bacterial sensitivity of antibiotics. *Edin. Med. J.* **59**: 178.
- Madan, M.M., R.S. Singhal, and P.R. Kulkarni. 1996. An approach into the detection of authenticity of black pepper (*Piper nigrum* L.) oleoresin. *J. Spices Arom. Crops.* **5** : 64-67.
- McLaughlin, J. L., and L. L. Rogers. 1998. The use of biological assays to evaluate botanicals. *Drug Information J.* 32:513-524.
- Nadkarni, A. K., and K. M. Nadkarni. 1954. *Indian Materia Medica.* Vol. 2. Popular Book Depot, Bombay.
- Paradkar, M. M., R. S. Singhal, and P. R. Kulkarni. 2001. A new TLC method to detect the presence of ground papaya seed in ground black pepper. *J. Sci. Food Agric.* **81** : 1322-1325.
- Parmar, V.S., S.C. Jain, K.S. Bisht, R. Jain, P. Taneja, A. Jha, O.D. Tyagi, A.K. Prasad, J. Wengel, C.E. Olsen, and P.M. Boll. 1997. Phytochemistry of the genus *Piper*. *Phytochemistry* **46**: 597-673.
- Sengupta, S., and A.B. Ray. 1987. The chemistry of *Piper* species : a review. *Fitoterapia* **58** : 147-166.
- Takao, T., F. Kitatani, N. Wanatabe, A. Yagi, and K. Sakata. 1994. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shell fish. *Biosci. Biotech. Biochem.* **58** : 1780-1783.

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Table 1

Antimicrobial activity of various sequential extracts of *Piper nigrum* and its adulterants.

Test organisms		Sequential Extracts																			
		<i>P. nigrum</i>					<i>C. papaya</i>					<i>E. ribes</i>					<i>L. camara</i>				
		PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ
<i>Bacteria</i>																					
<i>Pseudomonas aeruginosa</i>	IZ [†]	9	7	-	-	-	-	7	8	10	±	-	-	10	13	10	-	7	8	-	
	AI*	0.52	0.41	-	-	-	-	0.41	0.47	0.58	±	-	-	0.50	0.76	0.58	-	0.41	0.47	-	
<i>Escherichia coli</i>	IZ	11	9	12	10	±	18	13	7	8	11	±	±	8	10	15	9	7			
	AI	0.61	0.50	0.66	0.55	±	1.00	0.72	0.50	0.33	0.61	±	±	0.44	0.55	0.83	0.50	0.38			
<i>Enterobacter cloacae</i>	IZ	-	8	-	10	-	-	-	-	-	9	±	-	-	12	7	-	7	-	-	
	AI	-	0.57	-	0.71	-	-	-	-	-	0.64	±	-	-	0.85	0.50	-	0.50	-	-	
<i>Staphylococcus aureus</i>	IZ	15	11	15	7	-	10	10	8	-	9	-	-	10	10	11	12	-	-	-	
	AI	2.14	1.57	2.14	1.0	-	1.40	1.40	1.14	-	1.20	-	-	1.40	1.40	1.50	1.70	-	-	-	
<i>Fungi</i>																					
<i>Penicillium crysogenum</i>	IZ	15	16	15	7	-	-	8	-	10	9	-	-	±	-	-	13	-	-	-	
	AI	0.45	0.48	0.45	0.21	-	-	0.24	-	0.30	0.27	-	-	±	-	-	0.39	-	-	-	
<i>Trichophyton rubrum</i>	IZ	15	18	-	12	-	12	-	10	12	9	8	-	-	-	-	10	-	-	9	
	AI	0.42	0.51	-	0.34	-	0.34	-	0.28	0.34	0.28	0.22	-	-	-	-	0.28	-	-	0.25	
<i>Candida albicans</i>	IZ	20	10	12	13	±	9	12	8	13	10	12	7	±	-	±	9	-	-	-	
	AI	1.80	0.90	1.09	1.18	±	0.81	1.0	0.72	1.18	0.90	1.20	0.63	±	-	±	0.81	-	-	-	
<i>Aspergillus niger</i>	IZ	12	18	14	16	-	-	-	9	-	7	-	-	-	-	-	10	-	-	12	
	AI	0.75	1.10	0.87	1	-	-	-	0.56	0.43		-	-	-	-	-	0.62	-	-	0.75	

[†] IZ = Inhibition zone (in mm) including the diameter of disc (6 mm).

(+) Trace activity

* Activity Index = $\frac{\text{Inhibition area of test sample}}{\text{Inhibition area of the standard}}$

(-) Not measurable activity

Abbreviations : PE = Petroleum ether; C₆H₆ = Benzene; CHCl₃ = Chloroform ; EtOH = Ethanol; AQ = Aqueous.

Standards : Mycostatin = 100 unit / disc.; Gentamycin = 10 µg/disc.

Table 2

Antimicrobial activity of various sequential extracts of different *Piper* species

Test organisms	Sequential Extracts															
	<i>P. longum</i>					<i>P. chaba</i>					<i>P. betle</i>					
	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	PE	C ₆ H ₆	CHCl ₃	EtOH	AQ	
Bacteria																
<i>Pseudomonas aeruginosa</i>	IZ+	10	11	8	10	11	15	18	12	14	8	10	8	6	7	8
	AI*	0.58	0.64	0.47	0.58	0.64	0.88	1.05	0.70	0.82	0.47	0.58	0.47	0.35	0.41	0.47
<i>Escherichia coli</i>	IZ	9	10	7	-	±	10	11	12	16	7	12	9	-	-	7
	AI	0.50	0.55	0.58			0.55	0.61	0.66	0.88	0.38	0.66	0.5			0.38
<i>Enterobacter cloacae</i>	IZ	7	7	14	8	-	-	11	-	-	7	8	7	6	-	9
	AI	0.50	0.50	1.00	0.57			0.78			0.50	0.57	0.50	0.42		0.64
<i>Staphylococcus aureus</i>	IZ	6	±	-	7	±	8	17	13	-	±	9	7	6	9	8
	AI	0.35			0.41		0.61	1.0	1.30			0.52	0.41	0.35	0.52	0.47
Fungi																
<i>Penicillium crysogenum</i>	IZ	12	-	±	-	7	14	±	-	±	-	13	8	-	±	18
	AI	0.36	-			0.21	0.42					0.39	0.24			0.54
<i>Trichophyton rubrum</i>	IZ	12	9	-	9	-	15	7	15	-	-	12	-	-	6	±
	AI	0.34	0.25		0.25		0.42	0.20	0.42			0.34			0.17	
<i>Candida albicans</i>	IZ	-	±	-	9	8	22	15	10	±	-	18	-	10	8	-
	AI				0.81	0.72	2.0	1.36	0.90			1.63		0.90	0.72	
<i>Aspergillus niger</i>	IZ	14	-	-	6	±	6	±	-	-	7	9	±	-	±	-
	AI	0.87			0.37		0.37				0.43	0.56				

† IZ = Inhibition zone (in mm) including the diameter of disc (6 mm).

(+) Trace activity

* Activity Index = $\frac{\text{Inhibition area of test sample}}{\text{Inhibition area of the standard}}$

(-) Not measurable activity

Abbreviations : PE = Petroleum ether; C₆H₆ = Benzene; CHCl₃ = Chloroform ; EtOH = Ethanol; AQ = Aqueous.

Standards : Mycostatin = 100 unit / disc.; Gentamycin = 10 µg/disc.